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UNIVERSITY OF ALBERTA

Studies of Dieback and Canker Disease of Saskatoon Caused by *Cytospora leucostoma*

by

© Regina Ann Pluim

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

PLANT PATHOLOGY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

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ABSTRACT

Leucostoma persoonii (Nitschke) Hohnel [anamorph = Cytospora leucostoma (Pers.:Fr.) Sacc.] is a pathogen known to cause early dieback and canker disease on a variety of fruit trees in North America. It has recently been recognized as an economically important disease on Amelanchier alnifolia Nutt. in Alberta, Canada.

A survey of twelve commercial orchards throughout the province in 1988 and 1989 indicated that the disease was widespread and occasionally serious. Identification of the pathogen was based on cultural, morphological and physiological characteristics. Inoculation and reisolation of C. leucostoma from A. alnifolia confirmed Koch's postulates. Isolates from five locations were studied in detail to determine the extent of strain variability and to investigate mechanisms of pathogenesis. Differences among isolates were found to be significant with respect to cultural characteristics on various media. Growth responses of the isolates to a range of temperatures also varied significantly. Vegetative incompatibility was observed among isolates from different locations, indicating the presence of genetically distinct strains of C. leucostoma.

Potassium permanganate (0.02 N) titrations of filtrates from cultures grown over a 24 day period in potato-dextrose broth confirmed that oxalic acid was produced by C. leucostoma. The amount secreted by individual isolates, as well as the time of onset, were significantly different. Bipyramidal crystals were isolated from cultures grown in potato-dextrose broth supplemented with 0.1% (w/v) calcium carbonate. Energy dispersive

x-ray microanalysis confirmed the presence of calcium in the crystals. Oxalic acid secretion and sequestration of calcium from host tissue may be an important factor in pathogenesis of C. leucostoma.

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CHAPTER I

LITERATURE REVIEW

INTRODUCTION

Amelanchier alnifolia Nutt., commonly known as the saskatoon or serviceberry, is becoming an increasingly important commercial fruit crop in Alberta, Canada. At present, there are 80 ha in production with 31 ha bearing fruit. The estimated annual cash value of the crop is $300,000 (Hausher, L. 1990. Personal Communication. Alberta Special Crops and Horticultural Research Center). The saskatoon is a native bush fruit of the Yukon, Northwest Territories, the Canadian prairies and the northern plains of the United States. It is well adapted to northern climates and can tolerate a wide range of soil types. The shrub attains a height of up to five meters (Fig. 1), produces white flowers in the spring, and bears fruit in clusters of six to twelve, ranging from 0.6 cm to 1.0 cm in diameter in wild and 1.6 cm in cultivated varieties (Fig. 2). The fruit is usually bluish-purple, but colors of white to red to almost black have been observed (Harris, 1972). Although the saskatoon is usually referred to as a berry, it is actually classified as a pome (Mazza et al., 1978; Olsen and Steeves, 1981; Stout, 1983).

Saskatoons were used by native Indians as a constituent of pemmican and by early settlers as a source of fresh fruit (Harris, 1972). The selection of saskatoons for domestication began in 1918 by Dr. W.D.

Fig. 1. Saskatoon orchard located at Brooks, Alberta. The bush attains a height of up to 5 meters.

Fig. 2. Fruit of Amelanchier alnifolia occurs in clusters of 6 to 12. While frequently referred to as a berry, the fruit is actually classified as a pome.

Albright, in Beaverlodge, Alberta (Graham, 1975). Since that time a number of cultivars have been developed from superior native genotypes. The most widely grown cultivars are 'Smoky' and 'Pembina' which are valued because of their superior fruit quality and prolific suckering habit.

With the increase in saskatoon production, diseases which may limit productivity have become a concern to growers. One disease of economic importance that appears to be increasing in incidence and severity is dieback and canker disease, caused by Leucostoma persoonii (Nitschke) Hohnel [= Valsa leucostoma (Pers. ex Fr.) Fr.]. However, the anamorph, Cytospora leucostoma (Pers.: Fr.) Sacc. is most commonly encountered in the field. Recently, the name Leucocytospora leucostoma (Pers.) Hoehn. has also been used for this anamorph (Hammer et al., 1989). This fungus is a well adapted wound pathogen and is known to be responsible for orchard decline in many fruit growing regions throughout North America.

Leucostoma persoonii and C. leucostoma have been reported to occur on saskatoons in fruit growing regions throughout Canada (Conners, 1967; Ginns, 1986) and the United States. Typical dieback symptoms on Prunus species in the Okanagan Valley, British Columbia have been found to be caused by C. leucostoma (Cujec, 1988). In Ontario, Cytospora canker is known to occur on plum, prune, sweet and sour cherry, apricot, nectarine, wild black cherries, choke cherries and apple (Anonymous, 1986). It is a particularly serious disease of peach. In the Niagara Peninsula it was shown that 98% of the trees in commercial peach orchards were affected by cankers that occurred on trunk, crotch, scaffold and bearing branches (James and Davidson, 1971). In Colorado, it is the most serious disease of peach, affecting 65% of the commercial crop (Luepschen et al., 1979).

Cytospora spp. have been isolated from sweet cherry orchards in Washington state, and in New York state this pathogen was found in association with cankers on sweet cherry trees that had been damaged by winter injury (Hildebrand, 1947; Kable et al., 1967; Regner et al., 1987).

Both L. persoonii and C. leucostoma were first observed on apple in Illinois in 1918 (Stevens, 1919). Since that time, this pathogen has been reported on apple in New Mexico (Leonian, 1921), Washington (Fisher, 1931), and Michigan (Proffer and Jones, 1989).

In Idaho, dieback and canker disease due to Cytospora spp. was recognized as a limiting factor in prune production in 1951. From 1953 to 1954, a number of extremely severe cases were found in fruit growing regions throughout the south western part of the state. Cytospora spp. were also isolated from diseased tissues of cherries, peaches, apricots, apples and willows (Helton and Moisey, 1955).

Dieback and canker disease caused by Cytospora spp. are also known to affect a wide variety of non-fruit hosts including sycamore, maple, pine, olive, elm, fir and many species of spruce (Helton, 1961; Kamiri and Laemmlen, 1981; Rumbos, 1988). Blue spruce is rarely killed by the disease but tree mortality has been reported for Englemann spruce and Douglas fir (Kamiri and Laemmlen, 1981). Numerous reports of Cytospora canker have also been made on poplar species (Biggs and Davis, 1983; Bloomberg and Farris, 1961; Filer, 1967; Schreiner, 1931).

SIGNS AND SYMPTOMS

Signs and symptoms associated with Cytospora infection on A. alnifolia indicate that the disease is similar to the Cytospora canker that is common on many stone fruits throughout North America. Symptoms of Cytospora diseases include drying and shrivelling of buds in the spring, flagging and shrunken bark, which eventually splits revealing reddish discoloration of the inner tissues (Davidson, 1986; Howard and Dykstra, 1986). Pycnidia and/or perithecia may be present on diseased branches (Pluim et al., 1989). A streak canker, similar to that caused by Cytospora spp. in Idaho prune orchards (Helton, 1956), has been reported in the Peace River region of Alberta. Internally, it spreads downward in a cone radiating out from the pith. Involvement of the cambium results in death to the terminal portions of the branch (Davidson, 1985).

ETIOLOGY

Leucostoma persoonii (Ascomycotina, Pyrenomycetes) belongs in the order Sphaeriales, with anamorphs in the genus Cytospora. Originally, these fungi were included in the order Diaporthales which consisted of all stromatic fungi. The teleomorphic structures are characterized by erumpent, pseudostromatic ascomata, each containing a group of immersed perithecia. The stromatic tissues are delimited by a conceptacle or black zone line (Dennis, 1978).

Historically, there have been numerous taxonomic revisions of the Diaporthales. The earliest accounts by Fries in 1823 were largely

descriptive, based on superficial appearance of the ascocarp. Fries recognized a number of species which he placed within the genus Sphaeria Haller ex Fr. In subsequent years, these species were assigned to various genera in the family Sphaeriaceae. It was at this time that the genus Valsa Fr., which originally included Leucostoma (Nits.) Hohnel, was recognized. Fuckel (1870) differentiated diaporthaceous genera based on the presence or absence of stromatal tissues and included Valseae among the families of diaporthaceous fungi with stromatic tissues. Karsten (1873) further subdivided the stromatic fungi into families and assigned Valsa to the family Valseae. In 1917, von Hohnel separated Leucostoma as a genus on the basis of the white disc, the long perithecial neck, and the well-developed entostroma surrounded by a compact darkened conceptacle (Barr, 1978). In 1973, Cytospora (Pers.:Fr.) Sacc. was recognized as the asexual stage of the genus Leucostoma (Muller and von Arx, 1973).

MORPHOLOGY

Leucostoma persoonii is a weak parasite on twigs and branches of a variety of fruit trees. The species is characterized by a stromatic area delimited by a dark marginal zone of tissue. The zone runs just beneath the periderm and bark surface so that the perithecia are embedded in an effuse entostromata with only a few remnants of the bark cells remaining. The disc is that portion of the stroma which is erumpent through the periderm and is composed of a thick interwoven layer of ectostromatic tissue. The perithecia are arranged circinately about a central disc and are monostichous (in a single layer). They are not usually found within

the same stroma as the pycnidia (Willison, 1936). Elongate beaks are formed that protrude through the disc and the ostiolar canal is lined with paraphyses (Wehmeyer, 1926). The perithecial centrum includes the asci, paraphyses and the inner portion of the perithecial wall. The asci are clavate, have a short evanescent base and are free within the perithecial centrum as a mass without any definite arrangement (Barr, 1978). A nonamyloid refractive ring at the apex of the ascus is characteristic of this genus. The asci typically contain eight, hyaline, allantoid, single-celled ascospores in a uniseriate arrangement. Ascospore measurements are 10 - 17 μm x 2 - 4.5 μm (Willison, 1936).

Pseudoparaphyses which arise prior to the development of asci are present within immature perithecia. It is believed that these filiform structures act as nurse tissue to developing asci. Thus, in mature perithecia there may be only remnants of these structures or they may be entirely absent (Wehmeyer, 1926).

Cytospora leucostoma (Deuteromycotina, Coelomycetes, Sphaeropsidales) has been identified as the conidial state by the repeated observation of teleomorphic and anamorphic stages in close association on natural substrates. Conclusive evidence, such as the development of one state from single spores of the other, is not available as it has not been possible to reproduce the sexual form in culture from either conidia or ascospores (Speilman, 1984).

The pycnidia are 1.0 to 1.5 mm in diameter and are characterized by labyrinthe-like locules composed of numerous interconnecting chambers arranged irregularly within a mass of ectostroma and uniting in the ostiolar region (Speilman, 1984; Sutton, 1980). The ostiole is single,

circular and prominent. The conidiophores are hyaline, septate, branch irregularly at the base and bear apical conidia. The conidiogenous cells are enteroblastic, phialidic and determinate. The conidia, which are exuded in a reddish-brown mucilaginous mass or cirrhus, are hyaline, aseptate, thin-walled, smooth and allantoid. They are 4.5 - 6.0 μm in length and 1.0 μm wide (Sutton, 1980).

PHYSIOLOGY

Mycelial growth rate, pycnidial formation and colony characteristics of Cytospora spp. in culture have been shown to be temperature dependent. Konicek and Helton (1962a) tested six isolates of Cytospora at temperatures ranging from 3 to 45 C. Growth was measured in four fixed directions until one of the cultures reached the edge of the plate. It was found that neither the growth rate nor the relationship of the rate of growth of one isolate to that of another at a given temperature was always the same. A wide range of colony characteristics was observed in response to temperature, rendering them unreliable as distinguishing factors among isolates.

Carbon sources were also found to have a marked effect on growth rate, sporulation habit and colony appearance (Konicek and Helton, 1962b). By varying the carbon source on which the Cytospora isolates were grown, Konicek and Helton (1962) concluded that the carbon source will have considerable influence on determining whether or not a fungus can be related to other isolates. Maltose was identified as the most desirable carbon source for Cytospora spp. resulting in excellent mycelial growth,

good production of pycnidia and fair conidial sporulation.

Significant differences in growth rate were also observed when various nitrogen sources were used (Konicek and Helton, 1962c). Pycnidial sporulation tended to vary inversely with mycelial growth. This is not surprising since nitrogen is associated with vegetative growth rather than reproduction. Konicek and Helton (1962c) found a lack of consistent relationships among Cytospora isolates in their ability to grow in the presence of different nitrogen sources. This suggested that different growth mechanisms may be present in different isolates and supports the notion that many isolates belong to different physiologic strains within a species (Konicek and Helton, 1962d). Overall, all Cytospora isolates tested responded poorly to urea, while potassium nitrate was considered to be the best source of nitrogen (Konicek and Helton, 1962c). Extreme variation in colony characteristics, surpassing those observed in previous temperature and carbon-source studies, was also noted.

Growth processes of Cytospora spp. have been found to have an acidifying effect on culture media (Konicek and Helton, 1962d). The pH optima for these fungi is approximately 5.5 and a growth induced increase in acidity of the media suggests fungal mechanisms that tend to modify the pH of the substrate in the direction of the optimum. Konicek and Helton (1962d) observed no prominent segregation of the isolates when the original pH was 4, 5, 6, 7, or 8. This provides evidence of the variability of individual isolates of the same Cytospora species. It may also explain the inconsistent virulence characteristics between isolates and the variations in symptomatology. Not only do Cytospora fungi tend to acidify their substrates, they are able to function over such a wide range of pH that

almost any plant material should be suitable as an initial substrate as far as pH levels are concerned (Konicek and Helton, 1962d).

Lukezic et al. (1965) examined major vitamin requirements for L. persoonii and determined that the isolates tested were stimulated by a combination of thiamine and biotin, while the response to myo-inositol was variable. Choline was stimulatory to all isolates except one. The best growth generally occurred when choline and myo-inositol were used in combination with thiamine and biotin. The pH level was found to significantly influence the quantitative response to the vitamins. The higher the pH, the greater the need for vitamins.

STRAINS AND STRAIN VARIATION

Isolate dependent virulence represented a major step in developing the concept of strains and strain variation among Cytospora isolates (Schwarz, 1922). Cultural characteristics of both the sexual and asexual stages of C. leucostoma have been found to be highly variable. Lukezic et al. (1965) found significant differences among colony characteristics from ascospores of L. persoonii originating from the same ascus. In some cases, the descriptions corresponded to prior descriptions of L. cincta (Fr.:Fr.) Hohn, while others corresponded to L. persoonii. In a study on the effects of Cytospora isolates on stone fruits, Helton and Konicek (1961) found that fungal reisolations from inoculated trees tended to develop triangular segments not present in the original cultures. Similar results were obtained by Helton and Moisey (1955) using isolates from diseased prune trees. In addition to triangular sectoring, the reisolates developed much

more erratic and rapid growth habits. Similar variation in cultural characteristics were also noted among isolates of C. chrysosperma (Pers.) Fr. Christensen (1940) found a wide variation in the general appearance of the mycelium, time of pycnidial formation, and number and size of pycnidia among seven isolates of this species.

Compatibility studies using isolates of L. persoonii and L. cincta from peach orchards showed that dark interaction zones occurred at the interface of advancing colonies when isolates from the same orchard were paired (Helton and Konicek, 1961). This indicated that several interaction groups were present among the isolates within the orchard. Adams et al. (1990) obtained similar results by pairing ascospores from the same perithecium, ascospores from different perithecia in the same canker and ascospores from different cankers at different locations within an orchard. It was found that a number of vegetative compatibility groups (VCG's) occurred within a single perithecium as well as between perithecia. In pairing 67 ascospores from a single canker, 12 VCG's were observed. The ratios of segregating VCG's suggest that multiple genes control vegetative compatibility and it was concluded that L. persoonii is heterothallic. These findings are consistent with results obtained for L. persoonii (Hammer, 1988) and L. cincta (Hammar and Adams, 1987; Proffer and Hart, 1988).

Vegetative compatibility reactions are the result of pairing two genetically distinct mycelia (Carlile, 1986; Coates et al., 1988; Gregory,1983). Typically, two types of reactions are possible. A dark zone line may form resulting from melanization and knotting of hyphae along the interface of two antagonistic cultures (Goldstein and Gilbertson,

1981). A second type of reaction is the barrage reaction in which paired colonies are separated by a zone of sparse mycelial growth and hyphal disruption that may be associated with pigment production. The reaction may vary within and between species with respect to the width of the inhibition zone, color and intensity of the pigment and development of aerial mycelium on either side of the interaction zone (Anagnostakis, 1983; Coates et al., 1985; Goldstein and Gilbertson, 1981). If isolates are genetically similar, a continuous mat of mycelium will result with no distinctive line of demarcation (Goldstein and Gilbertson, 1981; Puhalla and Hummel, 1983).

SOURCES OF VARIATION

In sexual reproduction, genetic variation is the result of plasmogamy, karyogamy and meiosis. This type of reproduction allows for the greatest variability as there is random segregation of genetically different gametes at meiosis. In heterothallic fungi, outbreeding is obligatory, thus a compatible partner is required for fertilization to occur. The mating condition may be controlled by two or more alleles at a single locus (bipolar multiple-allele heterothallism) or by a number of alleles at two loci (tetrapolar multiple-allele heterothallism). The advantage of heterothallism is that it increases outbreeding and variability (Moore-Landecker, 1982).

In non-sexual reproduction, variation may be the result of cytoplasmic inheritance, heterokaryosis or parasexuality (Agrios, 1978).

Heterokaryosis is the occurrence of more than one nuclear type within the same mycelium. Factors responsible for this include mutation in any bi- or multinucleate homonucleate cell, the formation of binucleate sexual spores or hyphal fusion with subsequent nuclear migration of unlike nuclei into a single cell (Agrios, 1978; Parmeter et al., 1963).

Evidence for heterokaryosis has been provided in studies in which homokaryons, each carrying a single allele for a nutritional deficiency, could be combined into a heterokaryotic individual capable of conspicuously greater growth than either of the constituent homokaryons (Allard, 1960; Burnett, 1976; Esser, 1967; Moore-Landecker, 1982; Webster, 1985). This type of phenomenon is known as complementation and can impart a wide range of physiological flexibility as well as phenotypic variation.

Studies on the mechanism of heterokaryotic growth in Verticillium dahliae Kleb. have shown that cultures which are both homokaryotic and heterokaryotic appear to exist coincidentally (Puhalla, 1971). It was found that most hyphal cells of the fungus were uninucleate except for a small number of binucleate anastomosed cells. The heterokaryotic cells appeared to be responsible for supplying necessary nutrients to the homokaryons. It was also found that diploid sectors arose from the anastomosed heterokaryotic cells that were morphologically different from the haploids. This same phenomenon has frequently been observed in cultures of Aspergillus niger van Tiegh. and Penicillium chrysogenum Thom. The diploid nuclei are heterozygous for all characteristics in which the two types of haploid nuclei present in the heterokaryon differ. The result is sectors of mycelium that are predictably different from either of the

two kinds of mycelia resulting from haploid nuclei (Allard, 1960; Pontecorvo, 1958).

Hyphal fusion also leads to the exchange of cytoplasm and extrachromosomal inheritance. This may be manifested by changes in virulence or in the ability to utilize nutritional substances (Agrios, 1978; Kendrick, 1985). During cytoplasmic exchange, the volume of cytoplasm that accompanies one nucleus may be much greater than the volume associated with the other nucleus. The result is that the offspring more closely resemble the parent that contributes the greater volume of cytoplasm. In Aspergillus glaucus Link, it has been determined that spore germination, growth rate, pigmentation and density of perithecia are under cytoplasmic control (Kendrick, 1985). Plasmids, or double-stranded extrachromosomal DNA, are known to be sources of cytoplasmically transmitted variation. Kistler and Leong (1986) have recently isolated plasmid-like DNA, believed to be responsible for host specialization, from races of Fusarium oxysporum Schlect. emend. Snyder and Hansen f. sp. conglutinans (Wr.).

The parasexual cycle involves recombination in the absence of sexual reproduction. Somatic hyphae fuse and exchange two unlike haploid nuclei, thus establishing a heterokaryon. The nuclei then fuse to form somatic diploids, followed by mitotic recombination and non-meiotic reduction to the haploid condition (Agrios, 1978; Kendrick, 1985; Pontecorvo, 1958; Webster, 1985). This condition is rare, occurring in fewer than one conidium in a million. However, it may still be an important factor in variation among fungi when considering the large numbers of conidia that are produced (Kendrick, 1985).

A number of significant differences exist between sexual and asexual reproduction. Sexual reproduction appears to be highly organized, genetically controlled and occurs in specialized organs. Parasexuality occurs randomly, does not appear to be genetically determined and does not require the presence of specialized structures. In sexual reproduction, crossing-over is likely to occur in every homologous pair of chromosomes during meiosis. During somatic recombination, crossing-over involves only a few chromosomes and occurs much less frequently (Kendrick, 1985).

EPIDEMIOLOGY

Of the sexual and asexual stages, Cytospora spp. are more commonly found in diseased orchards. Thus, conidia that occur throughout the year appear to be the primary source of inoculum, with ascospore release occurring mainly in the spring (Bertrand and English, 1976). Kamiri and Laemmlen (1981) indicate that this is also true for C. kunzei Sacc. and V. kunzei Fr., and that the numbers of ascospores and perithecia are small compared to pycnidia and pycnidiospores, suggesting that the ascosporic stage may develop several years after infection. Bertrand and English (1976) found only Cytospora spp. to be present in diseased orchards during the first three years of infection with the sexual stage appearing thereafter.

Spore release from pycnidia appears to be in response to wetting. The spores are exuded from pycnidia in a gelatinous cirrhus and are then disseminated by splashing and wind-blown rain (Bertrand and English, 1976; Luepschen and Rohrbach, 1969). Luepschen and Rohrbach (1969) found that

spore liberation in Colorado peach orchards was responsive to both relative humidity and temperature and that viable spores could be found on tree surfaces throughout the year. Dispersal gradients established for conidia indicate that the distance of dispersal and numbers caught at each distance are highly correlated with the mean wind velocity during rainfall.

Rohrbach and Luepschen (1968) found that cardinal temperatures for conidial germination of isolates taken from Colorado peach orchards were 4 to 10 C and 27 to 32 C, with maximum germination occurring at 27 C. Below 4 C, the conidia did not germinate and temperatures of 38 C were fungicidal. A period of 24 hours at the optimum temperature was required for maximum germination and infection to occur.

Germ tube development occurs most rapidly in a saturated atmosphere and appears to be slightly inhibited in free water. Rohrbach and Luepschen (1968) found that placing conidia in humidities below 100%, following incubation in a saturated atmosphere to allow germ tube development, was lethal to 50% of the population. This suggests that saturated conditions during the night, followed by dry periods during the day, may reduce infection by reducing the number of viable spores.

The mechanisms of ascospore release and dispersal are not as clear and a number of methods for release have been proposed. It has been suggested that asci become detached within the perithecium and the spores ooze out in a matrix somewhat less gelatinous than that produced by the pycnidia (Bertrand and English, 1969; Christensen, 1940). The ascospores then collect around the ostiole in white sticky masses. A second theory is that the asci are forced up the neck to the ostiole where they burst, discharging spores forcibly into the air (Christensen, 1940). Bertrand and

English (1976) found ascospores caught in traps following rain storms, but could not conclusively determine the exact mode of release as the spores could have been water-borne (oozing from the perithecia) or air-borne (forcibly ejected and then caught in the wind currents). However, ascospores were frequently trapped following cessation of rain, providing evidence for forcible discharge. Ascospore release is apparently related to rainfall, with an increase following rainy periods. There is no evidence of any diurnal effect on discharge.

THE ROLE OF PREDISPOSING FACTORS IN *CYTOSPORA* INFECTIONS

Since the earliest reports of *Cytospora* invasion of fruit trees, there has been confusion regarding the pathogenicity and virulence of the fungi involved. These conflicting reports have been attributed to variation in the pathogen, differences in host susceptibility and predisposing factors that may weaken trees, thus rendering them more susceptible to infection. In some cases the pathogen has been reported to be inherently virulent (Helton, 1956; Helton and Moisey, 1955) with no reference to predisposing factors. Other reports relate pathogenicity and virulence of *Cytospora* spp. to environmental factors such as drought and winter injury (Helton, 1961; Kable *et al.*, 1967; Schoeneweiss, 1981). A number of reports indicate that seasonal development of the host plays an important role in limiting canker development (Bertrand and English, 1976; Helton and Konicek, 1961; Jones and Luepschen, 1971). Environmental conditions in the fall, associated with leaf abscission, have also been cited as important factors leading to canker development (Tekauz and Patrick, 1974; Weaver,

1963; Willison, 1936). In addition, cultivation practices that prevent proper hardening off and/or result in mechanical wounds have also been implicated in the spread of the disease (Willison, 1936, 1933).

WINTER INJURY

Winter injury, which has been implicated as a contributing factor in canker disease of peach, plum and cherry trees, generally follows a rapid drop in temperature to below freezing levels following mild fall weather (Schoeneweiss, 1988). Thus, the tissues of woody plants are not acclimated, as this requires gradual exposure to temperatures at or below freezing for some time. A second type of winter injury, which affects the crown, is caused by cold temperatures following a warm period during which the crown becomes active (Willison, 1936). In this case, the injury can girdle the stem at the cambium, disrupting translocation of carbohydrates to the roots. Frost cracks can develop in the bark as the result of tensile strains developing during periods of very low temperatures. These low temperature injuries are common infection courts for Cytospora fungi which are efficient invaders of wounds and are particularly prevalent in northern latitudes where low temperatures are common (Helton, 1961).

The problem is compounded by cultivation practices that tend to delay winter hardiness thereby rendering the trees more susceptible to injury and invasion by Cytospora fungi (Willison, 1933). In New York state, low temperature injury to sweet cherry trees that were vigorous because of high levels of nutrition in the fall was found to result in Cytospora canker in the spring. The combination of reduced winter hardiness and severe low

temperatures were cited as the principal predisposing factors to disease development (Kable et al., 1967).

HOST RESPONSE

Late spring and early fall have been implicated as the time of greatest canker activity in peach and prune trees. Jones and Luepschen (1971) studied the behavior of cankers on peach trees inoculated with C. leucostoma. Measurements of canker length and width, wound gumming and swelling, circumference of healthy stems and the depth of the canker into the wound were made. It was determined that the greatest influence on canker enlargement was host activity. Stem activity, which commenced during the March - June period and progressed rapidly until September, was inversely related to canker development. This was in spite of the fact that ambient temperatures at this time approached the optima for mycelial growth of the fungus. It was concluded that host activity, rather than temperature, exerted the greatest influence on canker enlargement.

A similar study by Bertrand and English (1976) was conducted on French prune trees in California. In this case, the reaction of vigorous versus nonvigorous trees to a single isolate of C. leucostoma was examined as well as the seasonal development of Cytospora canker. The trees were inoculated monthly for a period of one year and canker lengths were measured after an incubation period of one month. Various factors that might affect canker development were also measured, including development of callus tissue, increase in trunk diameter and ambient air temperature. It was found that canker activity was limited during those periods when host growth was most

active in vigorous trees. In nonvigorous trees, the opposite was true and the greatest canker activity occurred during those periods which should have been conducive to host development.

The results of these studies indicate that canker caused by C. leucostoma is most severe during the dormant season when host responses are limited. Fall injuries, caused by rapid temperature decline, are more likely to be invaded by Cytospora fungi as the dry tissues are exposed for longer periods of time before the healing process commences in the spring (Willison, 1933). Chang et al. (1989) evaluated peach cultivars for resistance to L. persoonii infection, as well as cold hardiness, and found that host-pathogen interaction differed significantly in the spring and fall. Leucostoma persoonii was most virulent during the fall and cultivars with L. persoonii tolerance as well as cold hardiness were most resistant to infection.

DROUGHT

Prolonged drought increases the likelihood of invasion by wound pathogens. Schoeneweiss (1981) inoculated trees in various stages of wilt with Botryosphearia dothidea (Moug. ex Fr.) Ces. & de Not., a common cause of stem cankers on a wide range of woody hosts. It was found that a predisposing threshold of -12 to -13 bars (soil water potential) was required for fungal invasion to occur and that the extent of colonization increased with decreasing water potential.

Bertrand and English (1976) examined late season water deficits in relation to Cytospora canker development on French prune trees. Three treatments, consisting of no irrigation after August 4, intermediate (four irrigations) and wet (twice the amount of water), were used. Tree water status was measured weekly with a pressure chamber to determine tree water potential. The pressure end point was taken as the chamber pressure necessary to force internal leaf water to just wet the cut end of a petiole. Percent bark moisture was also monitored weekly. The trees were inoculated with C. leucostoma and examined monthly to determine longitudinal canker extension. Good correlation was shown between fall water status and canker development. There was no significant difference between the intermediate and wet regimes. The dry plots, however, showed significantly greater canker development, suggesting that fall moisture status may play an important role in canker development.

EFFECT OF PRUNING

It is believed that many Cytospora cankers originate from pruning wounds. The type of wound as well as the time of year of pruning have been shown to have a significant effect on the probability of canker formation (Biggs, 1989; Wilson, 1984). Willison (1933), observed that wounds made in early spring were less likely to result in canker formation than those made during the dormant season. A certain amount of drying out and killing of the living tissues surrounding the wound provides an ideal infection court for Cytospora invasion. The earlier in the dormant season the wound is made, the longer the period of drying out and the longer the period of wound exposure to infection. Healing of pruning wounds made at the beginning of the growing season occurs faster, as wound periderm is formed and callus is rapidly produced. As growth slows later in the season, healing may be retarded, resulting in incomplete callus formation and only partial healing (Willison, 1933). In saskatoons, it has been shown that vegetative maturity is reached in late May (Friesen, 1986). While this may be desirable and responsible for cold acclimation, pruning after this date may be conducive to canker formation by Cytospora fungi.

Pruning techniques have also been indicated as having an influence on canker development. Typically, small branches from fruit trees have been removed using a cut that is parallel to the larger subtending branch as it was believed that this promoted rapid healing of the wound. In a three year study in Ontario comparing stub, flush and collar cuts, Biggs (1989) found that collar cuts significantly reduced the incidence of necrosis and L. persoonii infection. Similar results were obtained by Wilson et al.

(1984) on peach trees in West Virginia after one year. This is believed to be due to a compartmentalization process in which both mechanical and biochemical barriers of the host limit the spread of the pathogen.

LEAF SCARS

The rate of leaf abscission and presence of leaf scars have been implicated as factors in Cytospora infections. Weaver (1963) established a correlation between rate of defoliation and canker susceptibility using weekly leaf counts from mid-September until complete drop in 26 peach varieties. Those varieties in which abscission occurred early tended to heal more quickly and had fewer cankers than those with delayed abscission. Results of previous studies in which leaves were artificially removed and leaf scars were inoculated with V. cincta suggested that a well developed periderm limited the spread of infection as compared to those trees in which natural leaf fall was allowed to occur (Willison, 1936). Dhanvantari (1982) found that both L. cincta and L. persoonii were responsible for nodal infections. However, L. persoonii was most virulent in the early fall when the warmer daytime temperatures were prevalent.

PATHOGENICITY

Cytospora canker is characterized by a softening and darkening of tissues beneath the bark. Initially, the dieback symptoms were thought to be due to a toxin produced by L. persoonii (Helton and Konicek, 1961; Helton and Moisey, 1955; Willison, 1936). If a toxin is responsible, it is believed to move acropetally, as disease symptoms occur in portions of the plant terminal to the canker (Helton and Konicek, 1961).

Since the fungus can be isolated from deep within xylem tissues of infected plants, disruption of xylem conductivity at infection sites has also been cited as contributing to pathogenesis (Tsakade, 1959). It has been suggested that metabolic products of C. leucostoma impart viscosity to the xylary fluid and interfere with cross channel transfer. Reduced concentrations of calcium in limbs distal to nongirdling cankers therefore suggests the possibility of xylem dysfunction, as calcium is transported within xylem vessels, or removal of xylary calcium at cankered sites (Hampson, 1973).

It has been suggested that oxalic acid, functioning synergistically with tissue macerating enzymes may play an active role in the pathogenesis of L. persoonii (Traquair, 1987). Polygalacturonase, cellulase, xylanase and phosphatidase have been found in variable amounts in Cytospora isolates. Gairola and Powell (1971) observed that virulent isolates were able to produce all four enzymes, with comparatively larger quantities of the cellulolytic, xylolytic and phosphatide-degrading enzymes. Those isolates that demonstrated poor ability to become established in host tissue also showed poor ability to produce these enzymes in vivo.

Traquair (1987) established the synthesis of oxalic acid and formation of calcium oxalate crystals by L. persoonii isolates from peach. Oxalic acid was detected using gas chromatography. Bipyramidal and prismatic calcium oxalate crystals similar to those described for Sclerotium rolfsii Sacc. (Punja and Jenkins, 1984) were found in both culture filtrate and peach bark tissue inoculated with L. persoonii. Oxalic acid acts synergistically with the polygalacturonases by lowering the pH of infected tissue, thus enhancing the activity of the cell wall degrading enzymes (Bateman and Beer, 1965; Punja and Jenkins, 1985; Stone and Armentrout, 1985; Traquair, 1987).

VIRULENCE

Cytospora spp. are known to be quite variable in their aggressiveness on hosts, ranging from vigorous wound pathogens to saprophytic organisms (Helton and Konicek, 1961). Gairola and Powell (1971) attributed the difference in virulence to the differential production of the cell wall degrading enzymes polygalacturonase, cellulase, xylanase and phosphatidase, with the more aggressive strains producing these enzymes in comparatively larger quantities.

Recently, virus-like particles have been identified in hypovirulent strains of L. persoonii (Hammar et al., 1989; Snyder et al., 1989). These particles are isometric, approximately 32 nm in diameter and are surrounded by a capsid sheath. They are associated with aberrant morphology of the pathogen, including reduced pigmentation, sparse mycelial growth, inability to sporulate and exudation from lysing hyphal tips (Snyder et al., 1989)

Hammar et al. (1989) identified nine segments of double-stranded RNA in nucleic acid extracts from an avirulent strain of L. persoonii using polyacrylamide gel electrophoresis. Using various curing procedures to remove segments of double-stranded RNA, isolates similar to the more virulent strains were obtained. This is the first evidence that virus-like particles in L. persoonii may play a role in virulence of the different strains.

OBJECTIVES

The objectives of this research were to: (1) Determine the incidence, severity and etiology of dieback and canker disease on Amelanchier alnifolia; (2) To examine and characterize variation among Cytospora leucostoma isolates; (3) To study the role of oxalic acid in the pathogenicity of C. leucostoma.

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CHAPTER II

INCIDENCE, SEVERITY AND ETIOLOGY OF EARLY DIEBACK AND CANKER DISEASE ON SASKATOONS

INTRODUCTION

In recent years, the production of saskatoons (Amelanchier alnifolia Nutt.) has been increasing throughout the prairie provinces. There are approximately 80 ha under cultivation in Alberta with 31 ha producing fruit. The estimated annual cash value of the crop is $300,000 (Hauser, L. 1990. Personal Communication. Alberta Special Crops and Horticultural Research Center, Brooks, Alberta).

Saskatoons are native to the northern prairies and are winter hardy. Dieback and canker disease has reportedly been increasing in orchards throughout Alberta (Davidson, J.G.N. 1988 Personal Communication. Agriculture Canada Research Station. Beaverlodge, Alberta). The disease appears to be similar to Cytospora canker which is known to be a limiting factor in the production of peach, prune, sweet cherry and apple (Helton and Moisey 1955; James and Davidson, 1971; Leupschen et al., 1979; Proffer and Jones, 1989; Regner et al., 1987). It is caused by the Ascomycetes Leucostoma persoonii (Nitschke) Hohnel. The sexual stage is relatively rare and the asexual stage [Cytospora leucostoma (Pers.: Fr.) Sacc.] is most commonly encountered in the field. This organism is an efficient wound pathogen and predisposing factors such as pruning and mechanical

wounds, low temperature injury and drought are thought to contribute to the incidence of the disease.

Signs and symptoms of dieback and canker disease on saskatoons include drying and shrivelling of buds and leaves in spring, flagging, shrunken bark which eventually becomes cracked and exfoliated, dying back of twigs and the presence of pycnidia and/or perithecia on diseased limbs (Howard and Dykstra, 1986; Pluim et al., 1989).

In addition to fruit trees, Cytospora species have been found in association with cankers on spruce, poplar, willow, elm, fir, olive, sycamore, maple and pine (Helton, 1961; Kamiri and Laemmlen, 1981; Rumbos, 1988). Many of these species are often used in windbreaks adjacent to orchards and it is not known whether they may harbor strains of Cytospora capable of infecting saskatoons.

In order to identify the causal organism and determine the extent and severity of the disease in Alberta, surveys were conducted during the summers of 1988 and 1989. Inoculation trials were carried out to verify Koch's postulates and host range studies were performed using 14 woody hosts common to Alberta. As a means of maintaining cultures for extended periods, the viability of the fungus was assessed for one year when stored in liquid nitrogen.

MATERIALS AND METHODS

Survey

Surveys were conducted in 12 cultivated orchards throughout Alberta in the summer of 1988 and in 11 orchards in 1989. The province was divided into 3 areas according to Alberta Agriculture Regional Divisions. These were the north central region, northeast region and the Peace River region (Fig. 1). Within each of these areas, 4 orchards were randomly chosen. A systematic sampling technique was used for each orchard in which every tenth bush was examined and rated as having the disease based on symptoms of dieback, exfoliation, canker, shrivelled bark, flagging and/or the presence of pycnidia. Disease severity was rated numerically according to the number of main branches affected: 0 = no disease; 1 = 1% to 25%; 2 = 26% to 50%; 3 = 51% to 75%; 4 = 76% to 100%. The data was subjected to analysis of variance.

Inoculation Trials and Host Range Studies

Diseased branches were collected from each orchard. Spore measurements, as well as morphological characteristics of the sporocarps, were used to identify the pathogen.

Isolates from 4 survey locations (Olds, Blackfalds, Sexsmith and Manning) as well as an isolate from the Alberta Special Crops and Horticultural Research Center at Brooks were used for inoculation trials and host range studies. Pieces of diseased twigs were surface sterilized with 0.4% NaOCl for 3 min and placed in moist sporulation

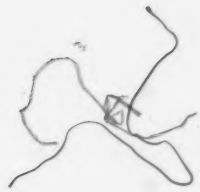


Fig. 1. Location of saskatoon orchards surveyed for dieback and canker disease in Alberta in 1988 and 1989.

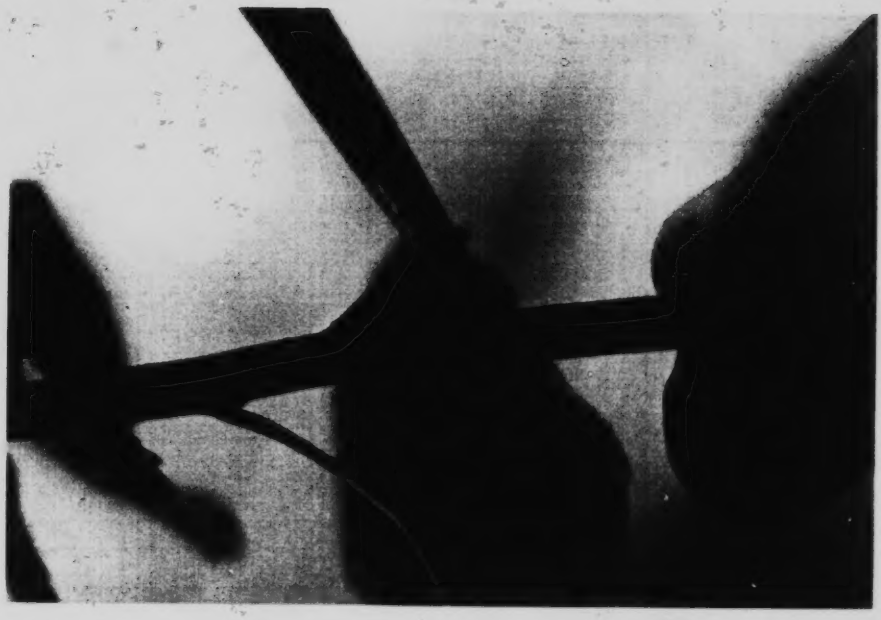
Manning
Sexsmith
Goodfare
Beaverlodge
Clyde
Lamont
Viking
Camrose
Blackfalds
Innisfail
Elnora
Olds

chambers overnight. Oozing pycnidiospores were then removed and placed in 4 ml sterile distilled water in a test tube and agitated to disperse the spores. An aliquot (0.1 ml) of this suspension was transferred to standard Difco potato dextrose agar and left at room temperature for 16 to 18 hr. Individual germlings were removed and transferred to malt agar. Sterilized toothpicks 0.5 cm in length were placed on top of the malt agar in plates containing the germlings. Within one week the mycelium had covered the plate and colonized the toothpicks.

For the greenhouse inoculations of saskatoons, one-year-old bushes (cv. 'Smoky') were inoculated with each of the 5 isolates selected. There were 6 replicates of each inoculation. Three bushes were planted per pot and the pots were arranged in a randomized complete block design. Sites on the bushes selected for inoculation were swabbed with 95% ethyl alcohol. Following evaporation of the alcohol, a piece of bark flap was aseptically cut to expose the cambium and xylem regions and sprayed with Histo Freeze (Fisher Scientific Company) to simulate freezing injury. A piece of colonized toothpick was placed on the wound, covered with the bark flap and sealed with parafilm (Fig. 2). The plants were maintained in a greenhouse at 22 C. They were watered and fertilized routinely. After 8 wk, the parafilm and toothpick were removed. Canker length was then measured and tissue samples were taken from the margins of the cankers. Following surface sterilization with 0.4% NaOCl, the tissue samples were plated on malt agar and maintained at 22 C for 3 wk. The presence of C. leucostoma was confirmed by comparing reisolates to the original cultures and by the presence of pycnidia with pycnidiospores. Data was subjected to analysis of variance and LSD for mean comparisons.

Fig. 2. Photographs depicting method of inoculation of Amelanchier alnifolia cv. 'Smoky' with 5 isolates of Cytospora leucostoma (Brooks, Olds, Blackfalds, Sexsmith and Manning). (a). Incision into bark tissue to expose underlying tissues. (b). Insertion of colonized toothpick under bark flap. (c). Wounds were wrapped with parafilm following insertion of the colonized toothick to prevent desiccation and provide a more optimal environment for fungal growth. (d). Following inoculation, wounds remained wrapped for 8 weeks.

a



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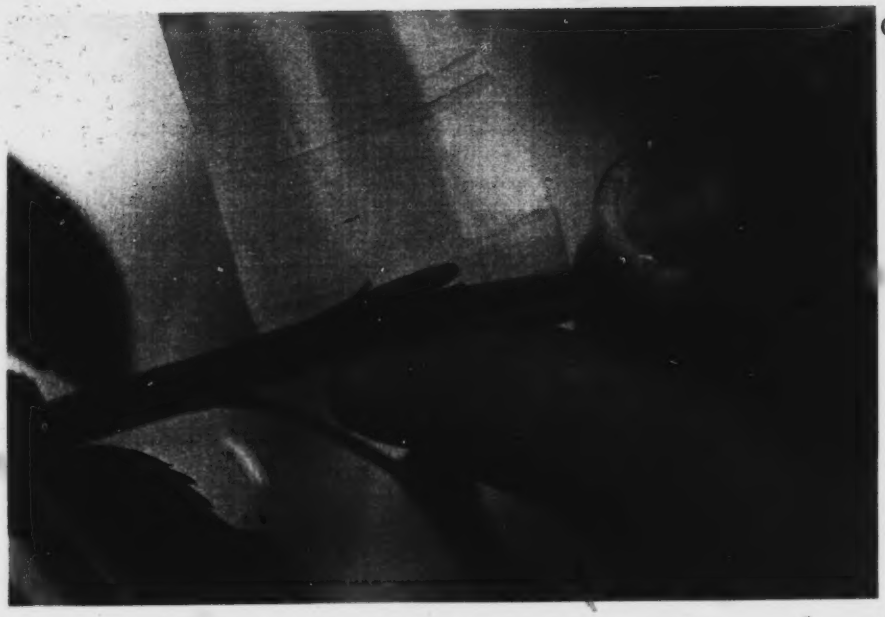

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d

Using identical techniques, 14 different woody species (one-year-old) were inoculated with three of the isolates (Brooks, Olds, Manning). These species included Pinus L. sp., Fraxinus pensylvanica lanceolata Borkh., Syringa vulgaris L., Acer L. sp., Picea A. Dietr. sp., Malus Mill. sp., Prunus serotina Ehrh., Populus x Brooks #6, Populus deltoides Bartr., Populus x Griffin, Cornus L. sp., Salix acutifolia L., Salix pentandra L. and Potentilla L. sp. Four replications were used. The trees were maintained under greenhouse conditions with routine watering and fertilization.

Inhibition Studies

The 5 isolates of C. leucostoma used in the inoculation trials were individually paired with Trichoderma Pers. sp. and Pestalotia de Not. sp. isolated from canker margins of inoculated saskatoon bushes. Five mm discs of mycelium were placed on opposite sides of malt agar petri dishes. Each isolate of Cytospora was paired with itself as a control. Ten replications were used for each pairing. The cultures were maintained at room temperature for two weeks and then examined to determine the effect of Trichoderma sp. and Pestalotia sp. on Cytospora in culture.

Storage of Cultures

The cultures were prepared from single spores and inoculated onto plates containing saskatoon decoction agar. The medium was prepared by boiling 250 g of saskatoon twigs in 1000 ml distilled water for 20 min and adding 15 g Bacto Agar. Once the plates were colonized, numerous pycnidia formed on the twig-pieces present in the medium. Thirty infected twig

pieces for each of the five isolates were aseptically removed and placed in labelled, sterile, polypropylene cryogenic vials. No cryoprotectant was used. The vials were cooled for two hours at 4 C, placed in a Handi Freezer tray (Union Carbide Model P/N R036-8C15) and suspended in the neck of a Union Carbide 35VHC Cryogenic Refrigerator that had been previously filled with liquid nitrogen at -196 C. Overnight exposure to the liquid nitrogen vapour allowed the cryotubes to cool at a rate of 6 C per minute. The following day the frozen cryotubes were loaded onto labelled canes and submerged into the liquid nitrogen. Vials containing 5 infected twig pieces were removed every 2 mo for one year. The tubes were thawed by immersing in warm water (30 C) for 20 min. The twig pieces were immediately placed on malt agar plates and the resulting colonies were assessed for viability, ability to produce pycnidia with pycnidiospores and colony characteristics.

RESULTS

Survey

Dieback and canker disease was observed in all orchards surveyed (Table 1). One orchard located in north central Alberta was not included in the analysis as it had not been taken care of for 5 yr and had an uncharacteristically high disease incidence. While no significant differences were noted between areas, the variability in disease incidence among orchards within an area was highly significant. The most commonly

Table 1. Incidence and severity of dieback and canker disease in 12 commercial saskatoon orchards surveyed in Alberta in 1988 and 1989. Bushes were rated according to the number of main branches affected: 0 = no disease; 1 = 1% to 25%; 2 = 26% to 50%; 3 = 51% to 75%; 4 = 76% to 100%

Area Surveyed	Incidence (%) (% of total bushes surveyed)	Severity (% bushes per category)				
		0	1	2	3	4
N. Central Alberta	28	72	20	5	1	2
N.E. Alberta	17	83	11	3	0	3
Peace River Region	13	87	11	1	0	1

observed symptoms were branch tip dieback (Fig. 3) and diffuse cankers. The cankers were most often located near the crown. They appeared as depressed areas with cracked and peeling bark. Removal of the bark revealed reddish-brown necrosis in the underlying wood. Sporocarps were often found in association with the cankers. Portions of the branch distal to the canker were frequently found to have numerous fruiting bodies of C. leucostoma and/or L. persoonii (Figs. 4-5). Cytospora leucostoma (asexual stage) appeared to be the primary source of inoculum in all orchards except one. In the northern most orchard located at Manning, Alberta, L. persoonii was predominant.

Inoculation Trials and Host Range Studies

Cytospora leucostoma was reisolated from 66 of the 90 plants inoculated. Other organisms most commonly isolated were Trichoderma sp. and Pestalotia sp. These organisms appeared to be antagonistic and either overgrew the C. leucostoma colonies or inhibited growth of the organism (Fig. 6a-b).

Differences in mean canker length among isolates were not significant (Table 2). Prolific callus formation was observed on all bushes. No wilting, flagging or formation of fruiting bodies was observed.

Inoculations of the 14 woody hosts resulted in only 3 infections. Two of these occurred on black cherry and one on Griffin poplar. No symptoms were observed on the Griffin poplar. The cankered area on black cherry appeared moist and discolored with extensive gummosis.

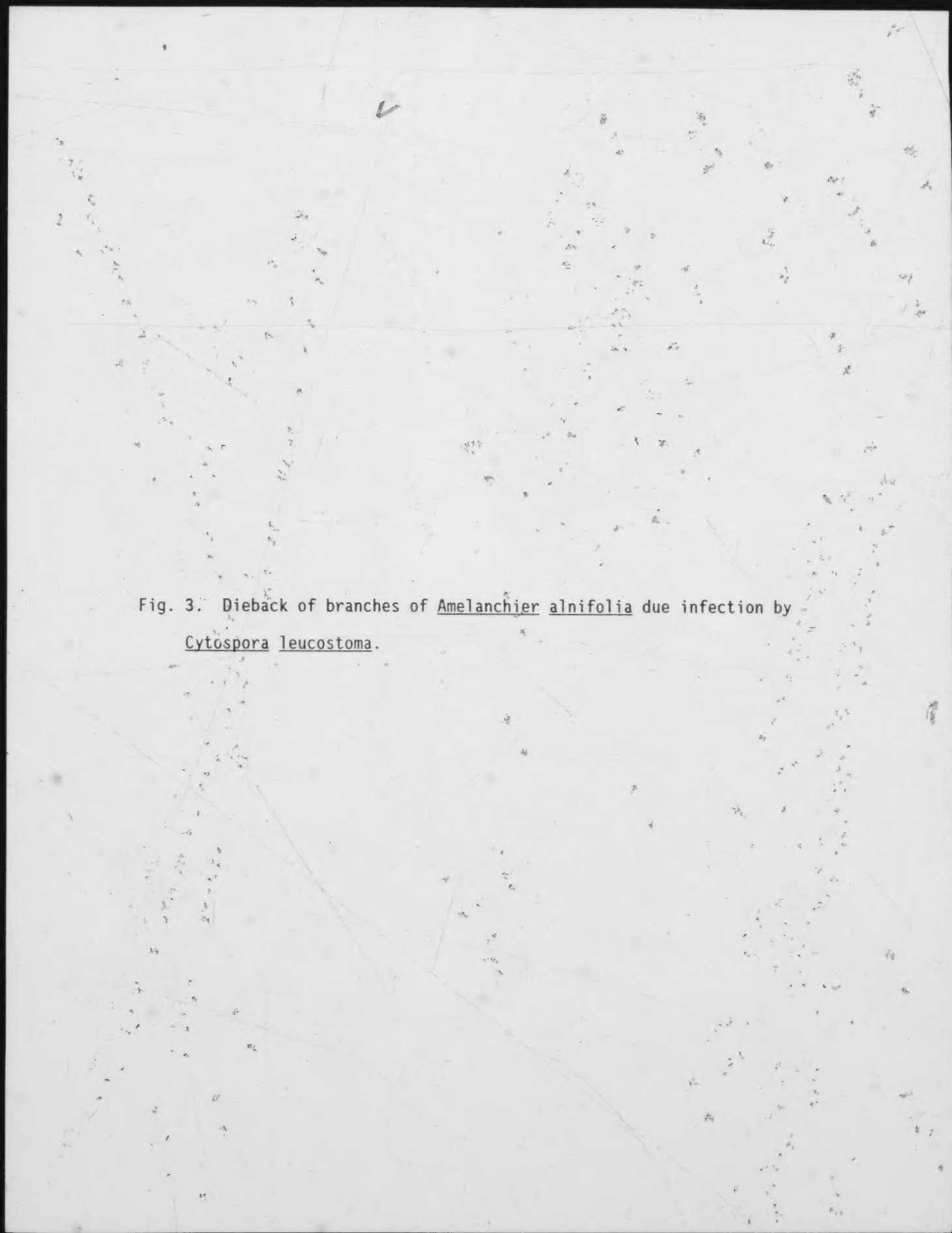


Fig. 3. Dieback of branches of Amelanchier alnifolia due infection by Cytospora leucostoma.

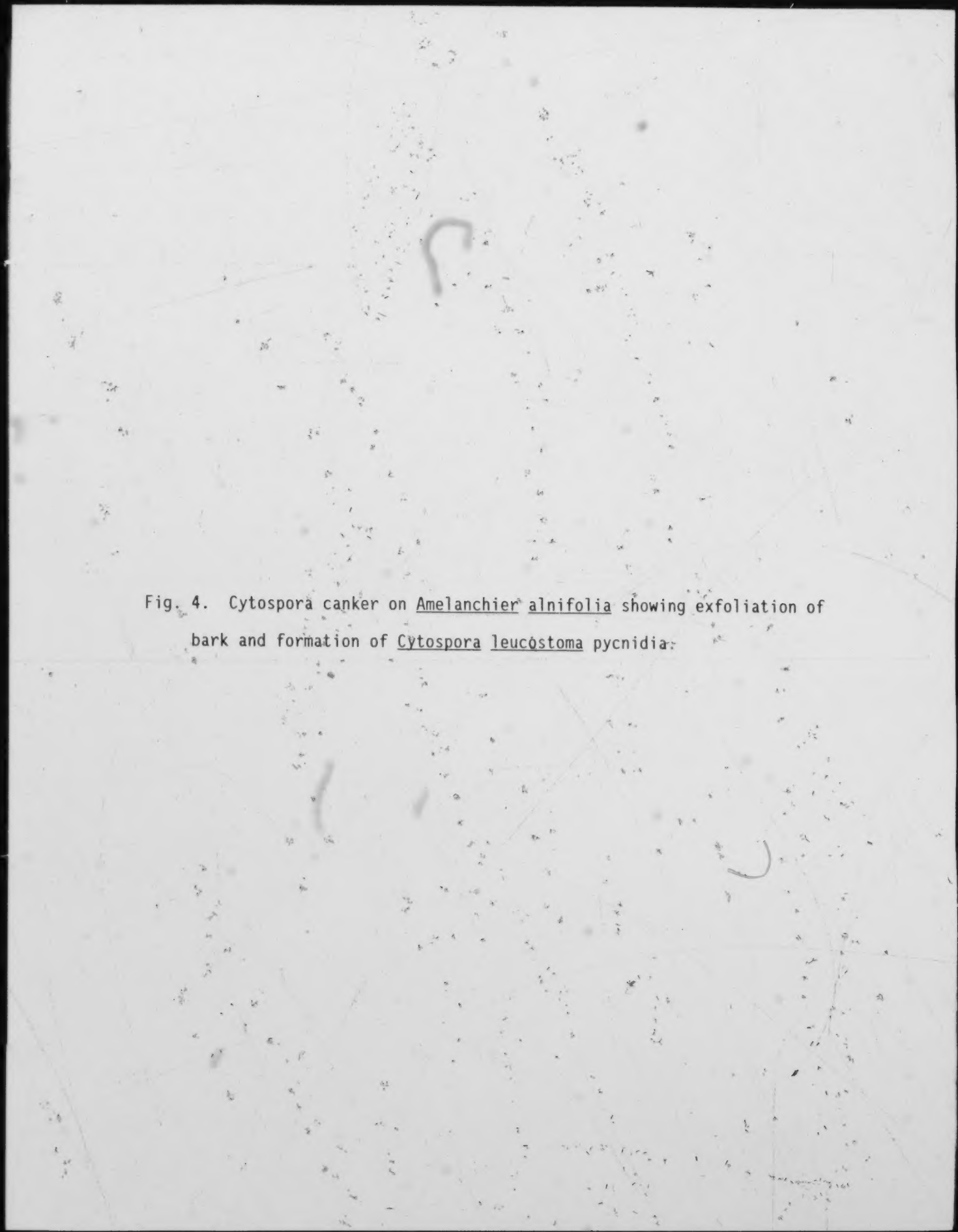
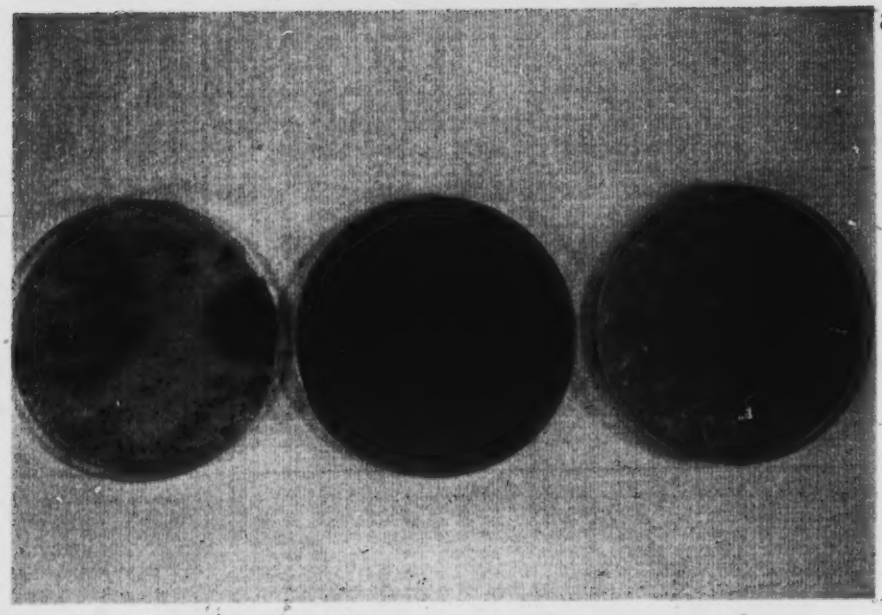


Fig. 4. Cytospora canker on Amelanchier alnifolia showing exfoliation of bark and formation of Cytospora leucostoma pycnidia.

Fig. 5. Pycnidial development of Cytospora leucostoma on diseased branches of Amelanchier alnifolia.

Fig. 6. Representative plates showing the interaction of *Cytospora leucostoma* isolates from Olds (a) and Blackfalds (b), with *Pestalotia* sp. (left), self (center), and *Trichoderma* sp. (right). In each plate, the colony on the left is *C. leucostoma*, while the colony on the right is *Pestalotia* sp. (left plate) and *Trichoderma* sp. (right plate).



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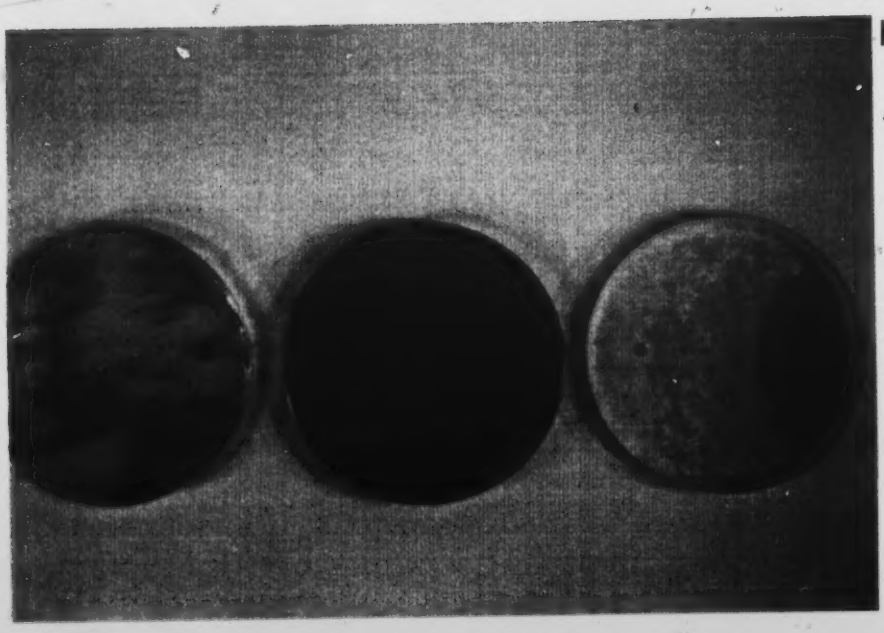

b

Table 2. Mean canker length on bushes of *Amelanchier alnifolia* cv. 'Smoky' artificially inoculated with isolates of *Cytospora leucostoma*.

Isolate	Mean Canker Length (cm)*
Brooks	1.67
Olds	1.51
Blackfalds	1.87
Sexsmith	1.85
Manning	1.61

*Mean of 18 bushes inoculated with each isolate.

Storage of Cultures

All isolates survived storage in liquid nitrogen for a period of one year. Colony characteristics did not deviate from the original cultures and pycnidia which had formed on the twig pieces prior to freezing began to ooze conidia in a reddish-brown cirrhus one week after plating. White mycelial growth could be seen radiating from the twig pieces within 48 hr. As in the original cultures, a dark brownish black pigment was produced after 2 wk by all the isolates except Brooks. Like the originals, these cultures appeared yellow-brown in color. Oozing pycnidia were formed on the surface of the mycelium. The spores were hyaline, allantoid and 5.9 - 6.2 μm x 1.2 μm which was within the range of the original cultures.

DISCUSSION

Dieback and canker disease was present in all orchards surveyed. No significant differences in disease incidence were noted between areas. However, variation in incidence among orchards within an area was highly signficant. This was most obvious in south central Alberta where two of the orchards were relatively disease-free while the remaining two showed a high incidence of disease as well as greater severity. These differences may be attributed to cultural practices. The healthy orchards were irrigated routinely while no irrigation was used in the diseased orchards. Prolonged drought is known to be a predisposing factor to fungal invasion by pathogens such as C. leucostoma (Schoeneweiss, 1981; Bertrand and English, 1976) and the extent of fungal invasion tends to increase with decreasing water potential (Schoeneweiss, 1981).

Inspection of diseased limbs indicated that dieback was also often associated with mechanical injury, pruning wounds or winter injury. This supports prior information relating Cytospora infections to predisposing environmental and physical factors (Helton, 1961; Helton and Moisey, 1955; Kable et al., 1967).

Winter injuries occurred when temperatures declined rapidly following prolonged periods of warm weather, resulting in a water soaked depressed area on the stems. The following summer, the bark appeared to be cracked and peeling with no callus formation. Cytospora was isolated from these sites and pycnidia or perithecia were frequently present on the terminal portions of the branch. Helton (1962) studied the effects of simulated freeze-cracking on invasion of dry-ice injured stems of Stanley prune trees

by naturally disseminated Cytospora inoculum and found Cytospora canker in association with wounds resulting from winter damaged tissues. Helton (1962) postulated that winter injury, and the resulting bark stresses due to irregularly distributed cambial growth in injured trees, creates numerous tiny wounds which provide ideal infection courts for invasion by Cytospora fungi.

Mechanical injuries were observed frequently at the base of diseased branches. Such injuries may become increasingly important as most saskatoons are now grown for commercial use and are mechanically harvested. Regardless of whether the wound appeared to be caused by winter injury or mechanical wounding, there was no evidence of callus formation. This supports Willison's (1933) observation that wounds incurred in late fall are less resistant to infection due to the lack of host response which can limit the spread of the fungus and may also have implications regarding pruning practices. Pruning in the late summer or early fall would not permit adequate wound healing and would increase the exposure period of dry damaged tissues to Cytospora invasion.

The C. leucostoma stage was almost always the primary source of inoculum. However, it was found in association with L. persoonii in two orchards (Olds and Manning). In the Olds orchard, the bushes were 20 years old and the disease was well established. Thus, it is possible that the conidial state had been present for a number of years prior to the appearance of the teleomorphic stage. Although it has been noted that the sexual stage does not usually appear until the disease has been present for two to three years (Betrand and English, 1976), the L. persoonii stage was found almost exclusively in the Manning orchard. No evidence was found to

indicate that the disease had been present for an extended period of time as this orchard was well maintained and the build-up of disease was highly unlikely. Thus, the reason for predominance of the sexual stage is not clear. It may be that environmental conditions contributed to this phenomenom as the Manning orchard was the northern most location of all orchards surveyed. Perhaps climate or photoperiod were contributing factors, although how this would affect the development of the sexual stage over the conidial state is not known.

Results from the inoculation trials suggest that no differences in pathogenicity on saskatoons existed among the 5 isolates tested. All isolates were significantly different from the controls indicating that the fungus was causing canker development. The strains of C. leucostoma isolated from saskatoon were not pathogenic to most of the woody hosts tested suggesting that these organisms may be host specific. Griffin poplar and black cherry were the only two species from which Cytospora was isolated following inoculation. Further studies would be required to determine conclusively whether or not these woody hosts are within the host range of these organisms.

The fact that only 66 out of 90 bushses inoculated showed Cytospora infection may have been due to the active growth of the host as evidenced by the prolific formation of callus at the canker margins. Jones and Leupschen (1971) have shown that host activity is an important limiting factor to Cytospora invasion in vigorous peach trees during the growing season, in spite of conditions that might be conducive to fungal growth. Also, tissue samples taken from the lateral margins of the cankers may explain the relatively low incidence of C. leucostoma reisolation. Helton

and Konicek (1961) demonstrated that when tissue samples were taken from the necrotic margins of cankers at the longitudinal extremes, the success of reisolating Cytospora from inoculated fruit trees was much higher than when samples were taken from the lateral margins.

In addition to C. leucostoma, Trichoderma sp. and Pestalotia sp. were the most frequently isolated organisms from inoculated saskatoons. Because these organisms grew very rapidly it was difficult to determine whether Cytospora was present in the canker, but was not detected because of masking by these other organisms. Results of the inhibition studies with Trichoderma showed that the Cytospora colonies ceased growing and were overgrown. Pestalotia grew very rapidly but did not overgrow the Cytospora colonies. It did however, limit mycelial growth so that expansion of the Cytospora colonies was inhibited. Whether true mycoparasitism exists cannot be determined from this study, although it does appear that both fungi are inhibitory to C. leucostoma in vivo. The exact nature of the interaction is not known and would require further microscopic examination. Komatsu (1976) reported that Trichoderma harzianum Rifai and T. polysporum Link ex Pers. coiled extensively around hyphae of Lentinus edodes (Berk.) Sing., destroying the host colony in culture. Electron microscope studies on the parasitism of phytopathogenic fungi by T. longibrachiatum Rifai indicated that infection papillae were found in the affected host cell wall (Hashioka and Fukita, 1969).

Storage in liquid nitrogen proved to be an effective method for storing C. leucostoma without the use of a cryoprotectant. All isolates had 100% survival after one year. Apparently the mucilaginous material within

the pycnidia acts as a cryoprotectant, thereby ensuring spore viability during freezing to temperatures as low as -196 C.

In conclusion, Cytospora canker appears to be widespread throughout Alberta in commercial saskatoon orchards. While this is not a new disease of saskatoons, increased intensity of crop production has resulted in a greater awareness of this as a potential problem in cultivated orchards. The incidence and severity of the disease may be related to cultural practices such as irrigation and time of pruning, or to practices that tend to delay hardening-off. Further investigations are required to conclusively determine the relationship of these factors to Cytospora canker.

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CHAPTER III

VARIATION IN ISOLATES OF *CYTOSPORA LEUCOSTOMA* ON *AMELANCHIER ALNIFOLIA*

INTRODUCTION

Dieback and canker disease is well known on peach, plum, sweet cherry and apple (Helton and Moisey, 1955; James and Davidson, 1971; Leupschen *et al.*, 1979; Proffer and Jones, 1989; Regner *et al.*, 1987). It is caused by *Leucostoma persoonii* (Nitschke) Hohn. [anamorph = *Cytospora leucostoma* (Pers.:Fr.) Sacc.] and *L. cincta* (anamorph = *C. cincta* Sacc.). These organisms are highly variable and the concept of strains and strain variation was recognized as early as 1922 (Schwarz, 1922). Colony characteristics in culture are often erratic and vary with temperature, carbon source, nitrogen source and pH of the media (Konicek and Helton, 1962a; Konicek and Helton, 1962b; Konicek and Helton, 1962c; Konicek and Helton, 1962d). A number of vegetatively compatible groups have now been recognized which may account for differences in virulence and pathogenicity among isolates (Adams *et al.*, 1990; Hammer *et al.*, 1989; Proffer and Jones, 1989).

In recent surveys, dieback and canker was reported to be present in saskatoon (*Amelanchier alnifolia* Nutt.) orchards throughout Alberta (Pluim *et al.*, 1990). While this is not a new disease of saskatoons, the increased intensity of crop production has resulted in a greater

awareness of the problem. Signs and symptoms of the disease on saskatoons include drying and shrivelling of buds and leaves in spring, depressed bark, dying back of twigs and the presence of pycnidia and/or perithecia on diseased branches (Howard and Dykstra, 1986; Pluim et al., 1989).

The objective of this study was to identify the causal agent, examine morphological and cultural characteristics and determine vegetative compatibility among groups.

MATERIALS AND METHODS

Taxonomic Studies

Collections of L. persoonii and C. leucostoma stages were made from diseased twigs chosen randomly from 5 orchards in Alberta. The presence of pycnidia and/or perithecia was determined using a dissecting microscope. Naturally infected host tissue was surface sterilized using 0.4% NaOCl and incubated overnight in a moist chamber to promote sporulation. Single spore cultures were obtained as described previously (Chapter 2; Etiology, Incidence and Severity of Dieback and Canker Disease on Amelanchier alnifolia) and transferred to malt agar. They were maintained in the dark at 22 C for 10 days. Colony characteristics were then examined and recorded. Morphological characteristics of the sporocarps were examined using a dissecting light microscope and a scanning electron microscope (SEM). Sporocarps were removed from twig surfaces with a scalpel and placed in a moist chamber to induce sporulation. After 24 hr they were fixed in 0.3% glutaraldehyde in Pipes buffer, dehydrated in a graded ethanol series and embedded in Epon 812. Sections 0.5 um were cut with a

Reichert OM U2 ultramicrotome and mounted on microscope slides. All sections were stained with acidified toluidine blue O. For SEM, resin removal was carried out, as described by Campbell et al. (1987), followed by critical point drying. A Cambridge 150 Stereoscan SEM was used to examine the material.

The length and width of conidia, ascospores and asci were measured at 400x with bright field microscopy. Perithecia and pycnidia were measured at 40x using a dissecting microscope.

Growth Media Studies

Cytospora isolates were taken from naturally infected stems collected at five Alberta locations (Brooks, Olds, Blackfalds, Sexsmith and Manning). Single spore cultures were obtained from pycnidia on 0.2 cm pieces of diseased twigs. The twig pieces were surface sterilized with 0.4% NaOCl for 3 min and placed in sporulation chambers overnight. Oozing pycnidiospores were then removed and placed in 4 ml sterile distilled water in a test tube and agitated gently to disperse the spores. This spore suspension was transferred to Difco potato-dextrose agar in 0.1 ml aliquots and left at room temperature for 16 to 18 hr. Single-spore germlings were then plated on 9 different media (PDA = Potato Dextrose Agar; SSDA = Saskatoon Stem Decoction Agar; CMA = Corn Meal Agar; MA = Malt Agar; LA = Leonian Agar; V-8A = V-8 Agar; OA = Oatmeal Agar; SLDA = Saskatoon Leaf Decoction Agar; LBA = Lima Bean Agar). The cultures were maintained in growth chambers at 22 C in the dark. Four replications were

used for each isolate. Radial growth was measured at 5 and 10 days. Growth pattern and colony characteristics were noted and growth rates compared.

Temperature Gradient Studies

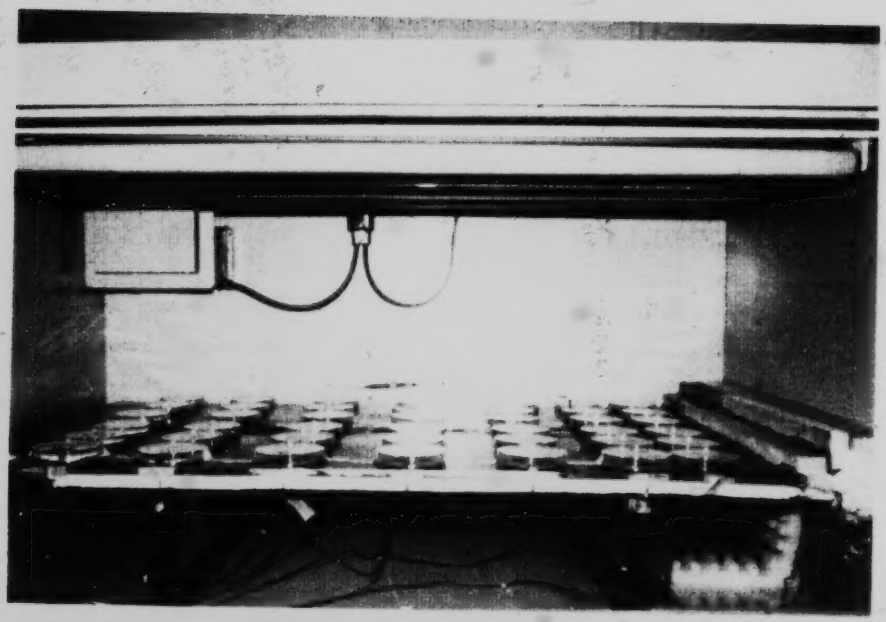
Five mm diameter discs were removed from the advancing colony margins of the 5 isolates used in the growth media study. Five replications of each isolate were plated onto malt agar. The isolates were then grown in a closed cabinet on a temperature gradient plate for 6 days. A probe inserted into water agar plates at each temperature was used to monitor the temperature of the agar. Temperatures were monitored continuously with a Honeywell DPR 1500 recorder (Fig 1a-b).

Two studies were carried out. In the first study, the colonies were grown at 4, 7, 11, 15, 18, 22 and 25 C. Colony diameter was measured in 4 directions after 6 days. In the second study, the colonies were grown at 20, 23, 27, 30, 33, 36 and 40 C. Colony diameter was measured in 4 directions daily for 6 days. Each study was repeated once.

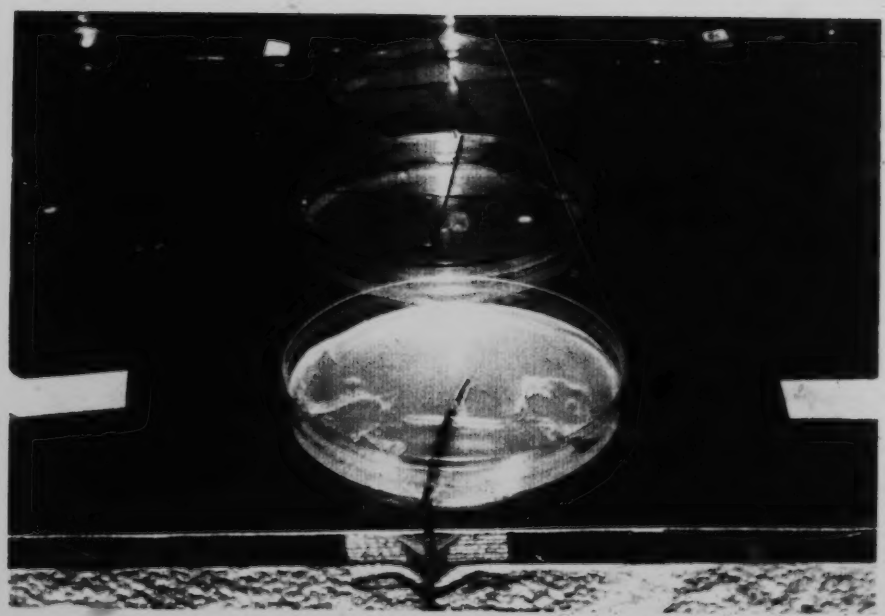
Vegetative Compatibility

Five mm diameter hyphal plugs were cut from the margins of 5 day old cultures grown on malt agar at 22 C. Each isolate was paired with itself and all other isolates by placing the 5 mm plug in the opposite quadrants of a 9 cm petri dish containing malt agar divided into 4 equal sections.

Fig. 1. (a). Temperature gradient plate used to monitor growth of Cytospora leucostoma isolates at various temperatures in culture. (b). Probe inserted in water agar for continuous temperature monitoring of media.



a



b

Ten replications were used. Vegetative compatibility was determined by observing the reaction along the line of contact between expanding colonies after two weeks. Colonies were considered compatible if a continuous mat of mycelium resulted with no distinctive line of demarcation. Colonies were judged incompatible if a dark zone line developed at the interface of advancing colonies or if a barrage reaction occurred. Isolates from the five locations as well as isolates from a single canker within an orchard were used and the experiment was repeated once.

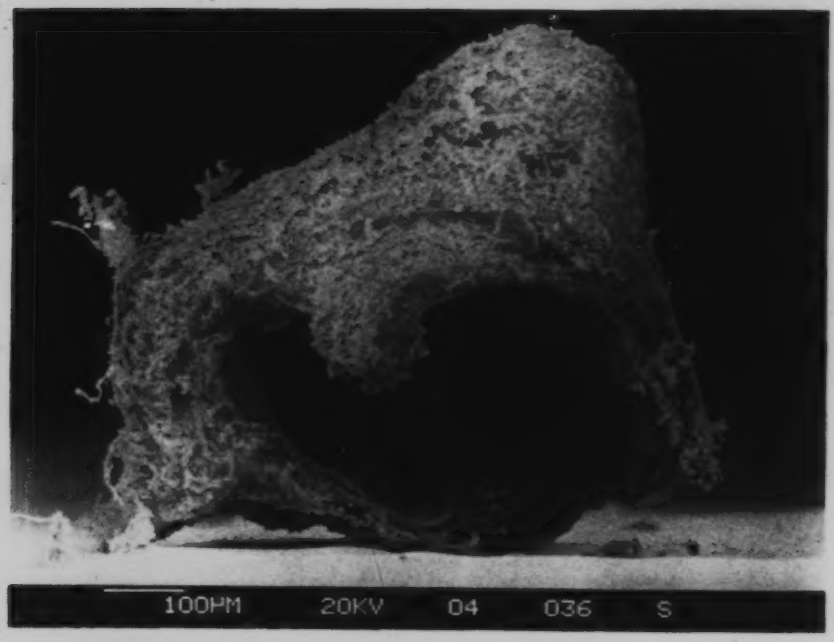
RESULTS

Taxonomic Studies

The anamorphic stage of the fungus was identified as C. leucostoma. The pycnidia were multilocular (Fig. 2) and the locules united in the ostiolar region. Each pycnidium had a single, circular and prominent ostiole emerging through the cracked bark of the host (Fig. 3). The long, cylindrical conidiophores were phialidic, determinate and hyaline with hyaline, aseptate, allantoid conidia formed at the apex of the conidiogenous cells (Fig. 4a-b). Conidia were discharged from the pycnidia in a reddish-brown cirrhus. The conidia ranged from 3.8 to 9 μm in length and 1.3 to 1.6 μm in width (Fig. 5) (Table 1). Identification of the isolates was confirmed by the C.A.B. Mycological Institute, Kew Surrey, UK (Appendix A) and by Dr. J. Traquair, Agriculture Canada Research Branch, Harrow, Ontario.

Fig. 2. Scanning electron micrograph showing the multilocular chambers of a pycnidium of Cytospora leucostoma isolated from Amelanchier alnifolia.

Fig. 3. Scanning electron micrograph of erumpent pycnidium of Cytospora leucostoma with a single ostiole on Amelanchier alnifolia.


100HM 20KV 04 036 S

194X 25KV WD:24MM S:00000 P:00008
200UM

Fig. 4a. Scanning electron micrograph of phialidic conidiophores within a pycnidium of Cytospora leucostoma.

Fig. 4b. Scanning electron micrograph of conidia of Cytospora leucostoma at the apex of phialidic conidiophores. Note the collar characteristic of phialides.

a

b

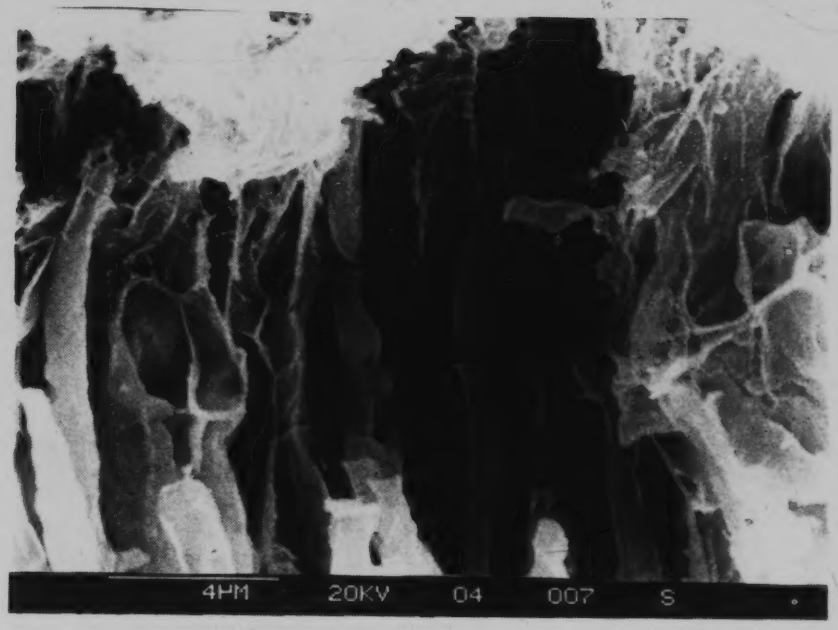


Fig. 5. Scanning electron micrograph of hyaline, allantoid conidia of *Cytospora leucostoma*.

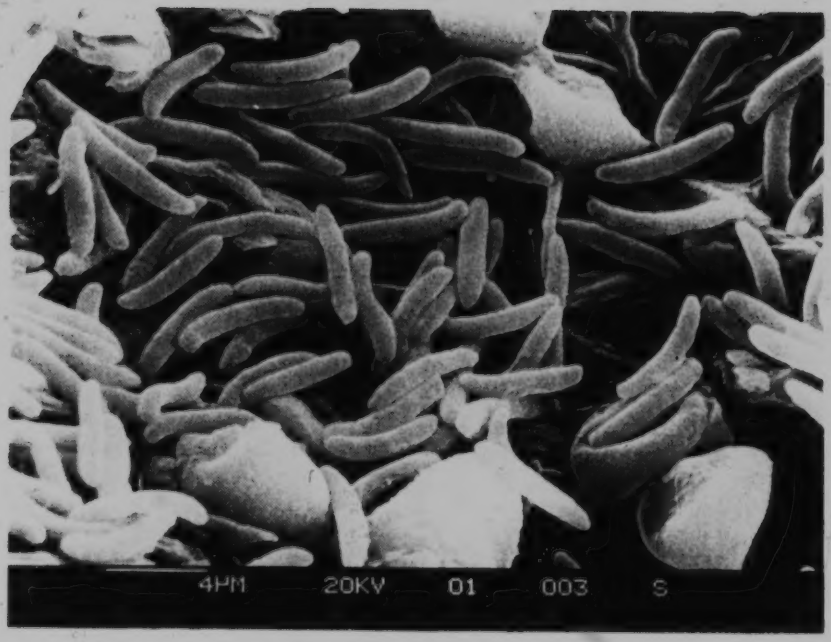

4PM 20KV 01 003 S

Table 1. Comparison of spore dimensions from 5 isolates of *Cytospora leucostoma* isolated from *Amelanchier alnifolia*.

ISOLATE	x LENGTH (μm)	RANGE (μm)	x WIDTH (μm)	RANGE (μm)
Brooks	6.2	4.5-9.0	1.5	1.3-1.6
Olds	6.0	4.5-8.2	1.5	1.4-1.5
Blackfalds	6.1	4.5-9.0	1.5	1.4-1.5
Sexsmith	6.1	3.8-9.0	1.5	1.5-1.6
Manning	6.1	4.5-9.0	1.5	1.3-1.6

Leucostoma persoonii was characterized by black perithecia with elongate beaks clustered in groups of 5 to 25 in a radial arrangement within a stroma (Fig. 6). The entire stroma was surrounded by a black zone line or conceptacle (Fig. 7). Remnants of paraphyses were observed in sectioned material. The asci were 36-60 μm in length and 9-12 μm in width and contained 8 hyaline, non-septate ellipsoid ascospores in a uniseriate arrangement (Fig. 8). Ascospores were 15-20 μm x 3 μm.

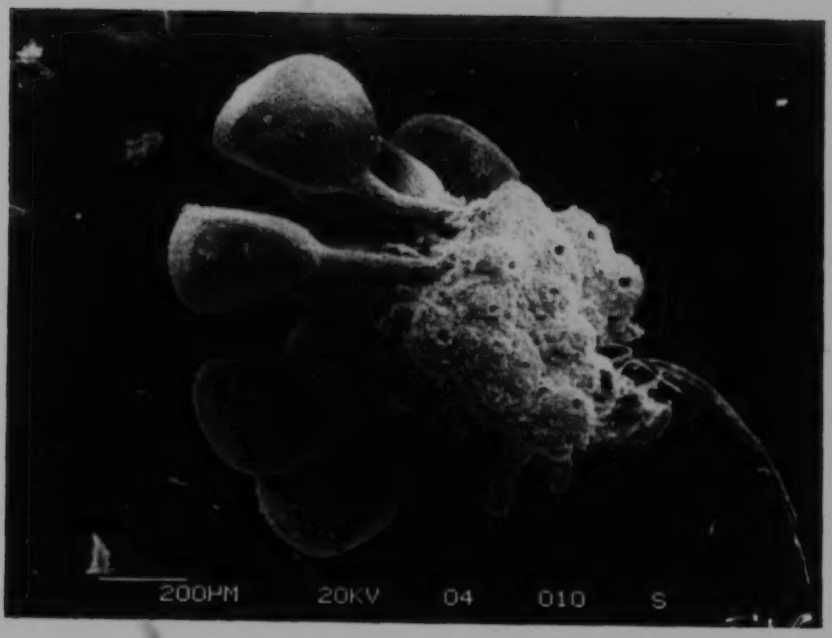
Growth Media Studies

Analysis of growth in culture indicated significant isolate x time and isolate x media interactions (Fig. 9). While all isolates behaved differently on different media (Fig. 10), the most consistent results were obtained with MA and SSDA. Saskatoon Stem Decoction Agar was the best medium for rapid sporulation, with pycnidia appearing after 5 days on the twigs placed in the agar. In MA, pycnidial formation was observed after 10 days. However, on this medium, colony appearance was variable among isolates (Table 2).

Vegetative Compatibility

A continuous mat of mycelium was formed when isolates from the same orchard were paired, with the exception of the Manning isolate (Fig. 11a-b). In this case, colony growth appeared to be limited with definite clear zones between each colony. Pairings between isolates from different locations resulted in the formation of dark zone lines at the interface of advancing colony margins, indicating that different vegetative compatibility groups existed at different locations (Fig. 12a-b).

Fig. 6. Scanning electron micrograph of a perithecial cluster of Leucostoma persoonii. Note the emergence of elongate beaks through well developed stromatic tissue.


200UM 20KV 04 010 S

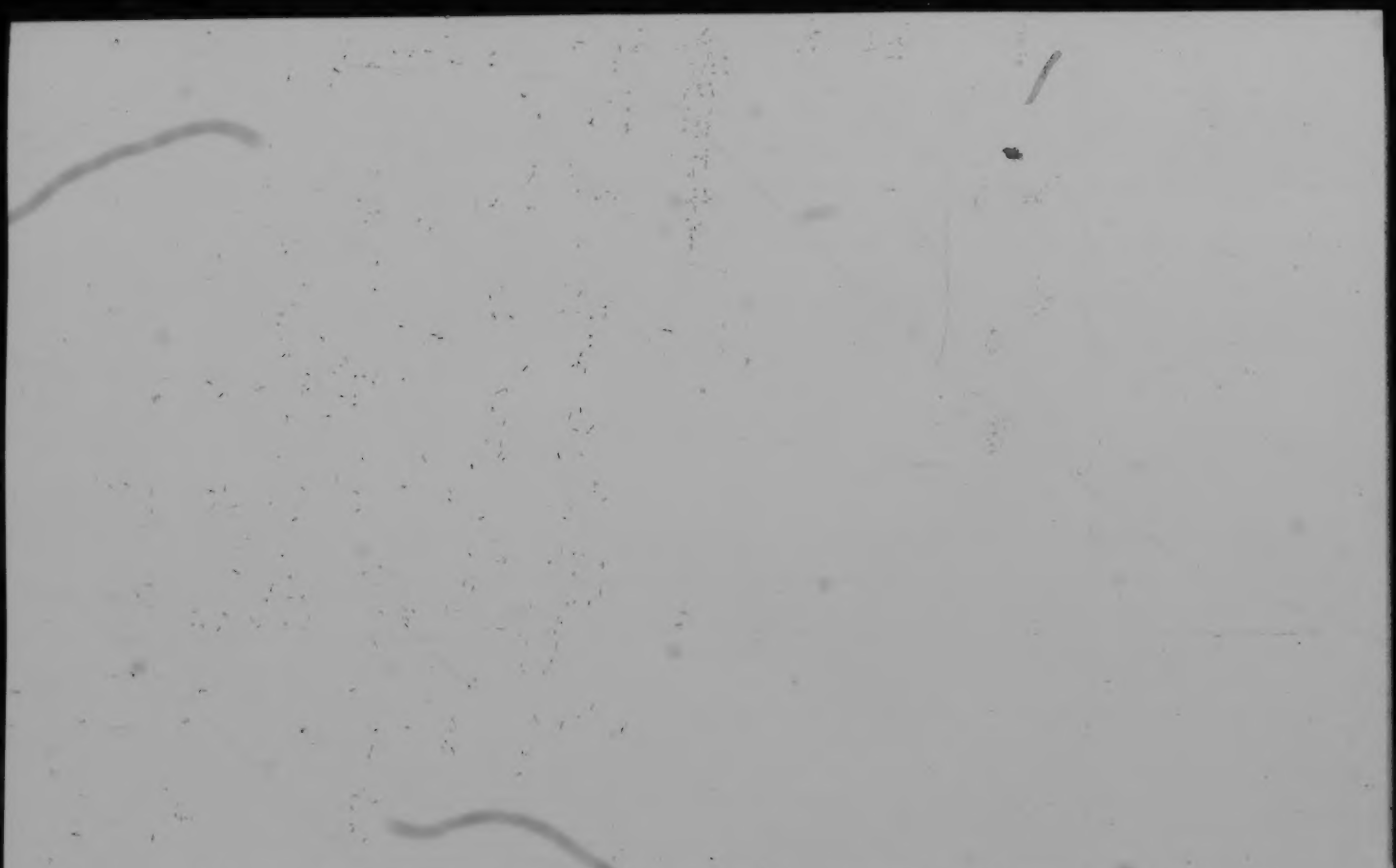
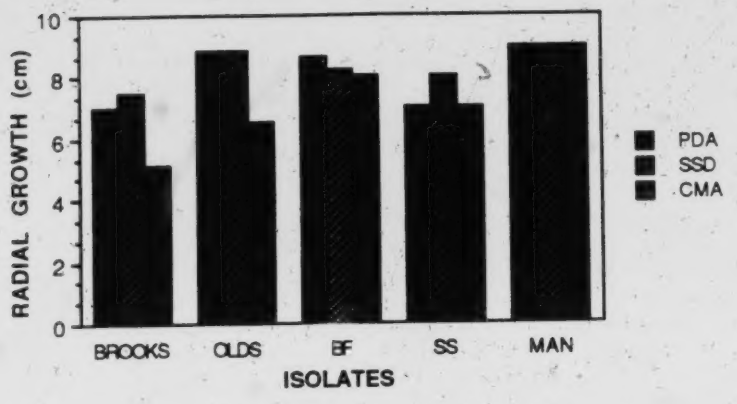
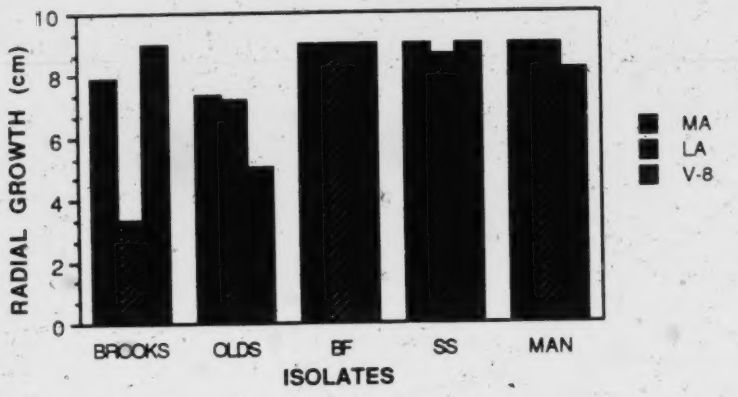


Fig. 7. An ascocarp of Leucostoma persoonii, exposed upon removal of bark. Note the radial arrangement of the perithecia surrounded by a black conceptacle.

Fig. 8. Cross-section of a perithecium of Leucostoma persoonii showing the club-shaped asci containing 8, hyaline, aseptate and allantoid ascospores in a uniseriate arrangement. Remnants of paraphyses can be seen among mature asci (mag. 400x).

Fig. 9. Growth of 5 Cytospora leucostoma isolates on Potato Dextrose Agar (PDA), Saskatoon Stem Decoction Agar (SSDA), Corn Meal Agar (CMA), Malt Agar (MA), Leonian Agar (LA), V-8 Agar (V-8A), Oatmeal Agar (OA), Saskatoon Leaf Decoction Agar (SLDA) and Lima Bean Agar LBA). Plates were incubated at 22 C and radial growth measurements were taken at 10 days. F-value for the interaction of isolate x media was significant at the 0.01 level.


RADIAL GROWTH (cm)
10
8
6
4
2
0
BROOKS
OLDS
BF
SS
MAN
ISOLATES
PDA
SSD
CMA


RADIAL GROWTH (cm)
10
8
6
4
2
0
BROOKS
OLDS
BF
SS
MAN
ISOLATES
MA
LA
V-8

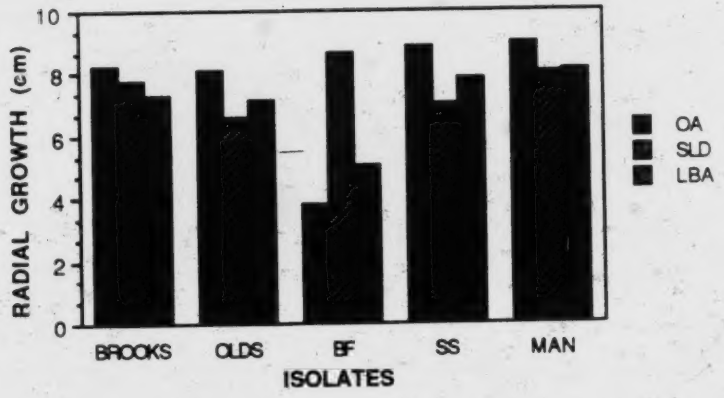

RADIAL GROWTH (cm)
10
8
6
4
2
0
BROOKS
OLDS
BF
SS
MAN
ISOLATES
OA
SLD
LBA

Fig. 10. Differences in cultural characteristics of a single isolate of Cytospora leucostoma (Brooks) on Potato Dextrose Agar (upper left), Malt Agar (upper right), Oatmeal Agar (lower left) and V-8 Agar (lower right).

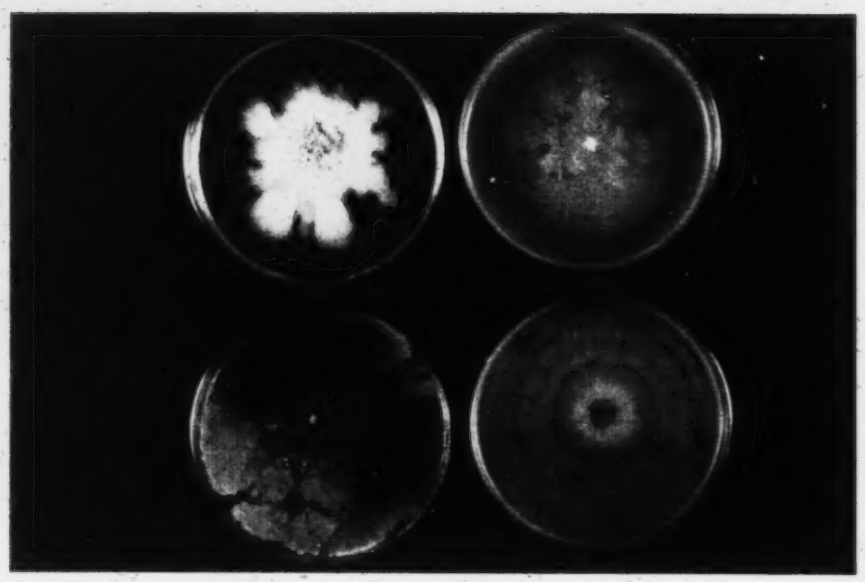
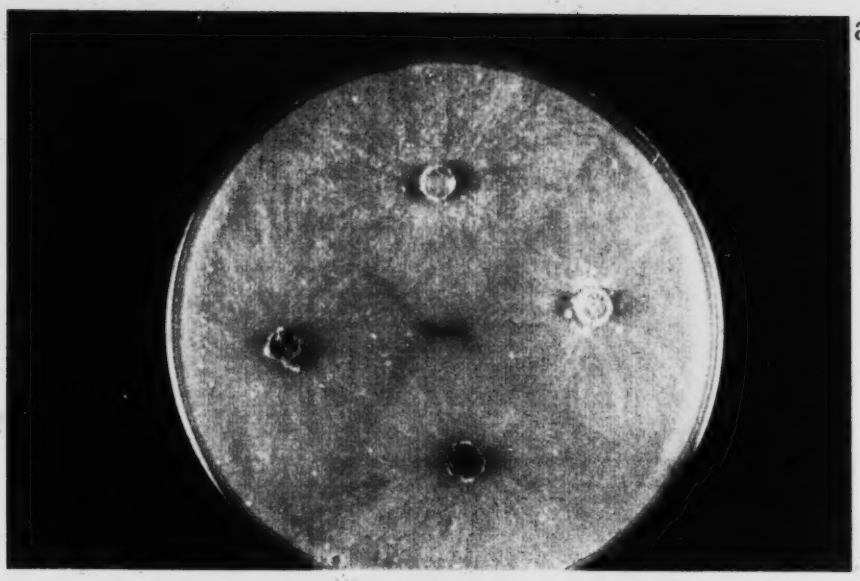


Table 2. Colony characteristics of 5 isolates of *Cytospora leucostoma* grown on Malt Agar for 10 days.

ISOLATE	COLONY COLOR (Upper)	(Lower)	SHAPE	MYCELIAL AND PYCNIDIAL CHARACTERISTICS
Brooks	White	Yellow	Variable, margins irregular or even	Fine, no aerial mycelium; Black, embedded pycnidia formed randomly in medium
Blackfalds	Yellow	Green	Radial growth with even margins	Fine, no aerial mycelium; Black, embedded pycnidia formed randomly in medium
Olds	White	Yellow	Radial growth with even margins	Fine, no aerial mycelium; Black, embedded pycnidia formed randomly in medium
Sexsmith	Grey	Black	Radial growth with even margins; sectoring common	Fine, no aerial mycelium; Black, embedded pycnidia formed randomly in medium
Manning	Grey	Brown	Margins irregular sectoring	Fine, no aerial mycelium Black, embedded pycnidia formed randomly in medium

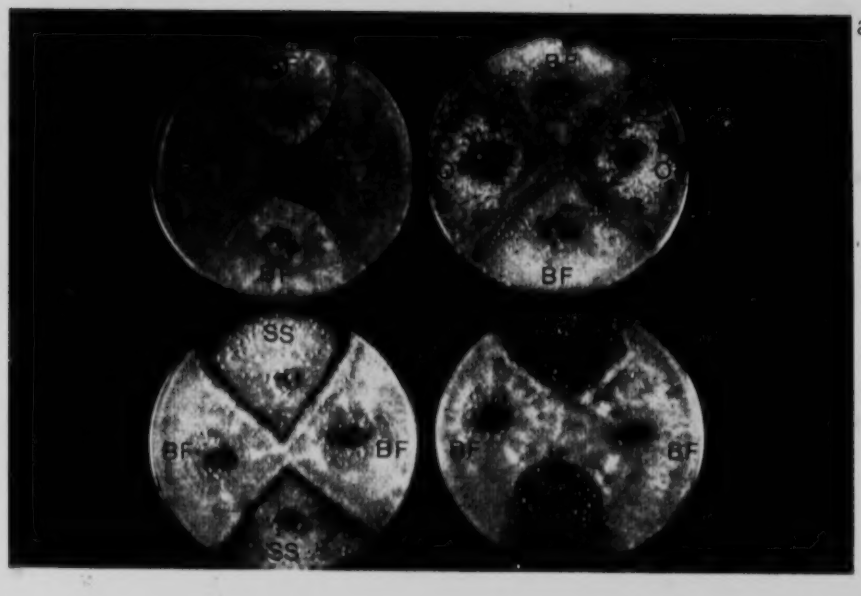
Fig. 11. (a). Representative plate showing a continuous hyphal mat characteristic of vegetative compatibility. Four isolates from a single canker on Amelanchier alnifolia located within the Brooks' orchard were inoculated onto Malt Agar and incubated at ambient room temperature (22 C) for 10 days. (b). Individual colonies of the Manning isolate maintained individual integrity regardless of the length of time in culture.

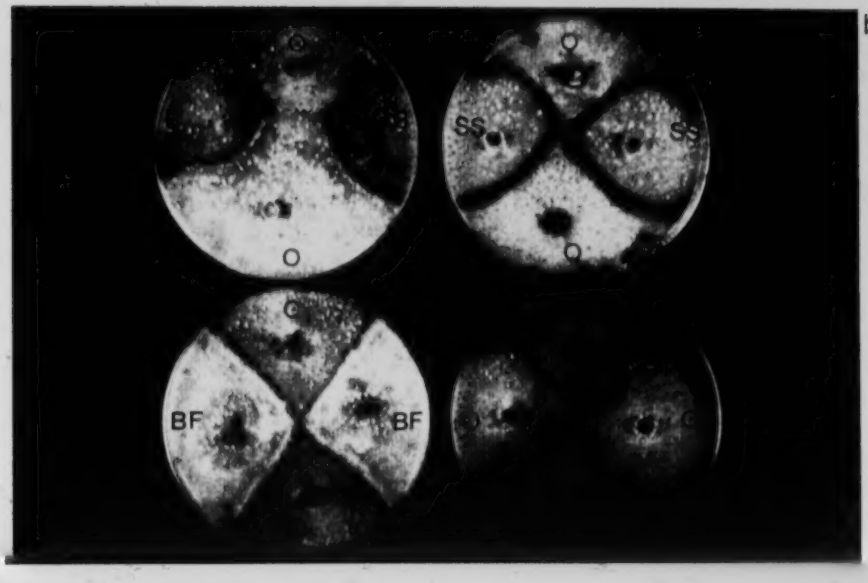


a

b

Fig. 12. Representative plates indicating dark zone lines formed along advancing colony margins of Cytospora leucostoma isolates grown on Malt Agar for 15 days at 22 C. (a) Blackfalds (BF) x Brooks (B) (upper left); Blackfalds x Olds (O) (upper right); Blackfalds x Sexsmith (SS) (lower left); Blackfalds x Manning (M) (lower right): (b) Olds x Brooks (upper left); Olds x Sexsmith (upper right); Olds x Blackfalds (lower left); olds x Manning (lower right).


a
BF
SS
BF
BF
SS
BF
BF

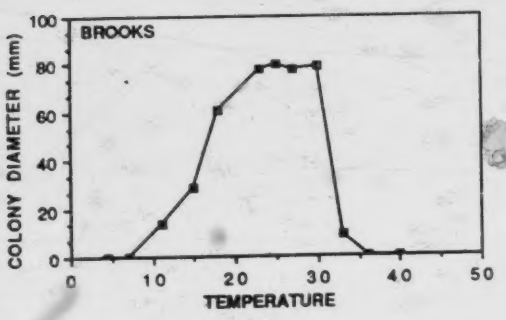

b
O
O
SS
SS
O
O
BF
BF

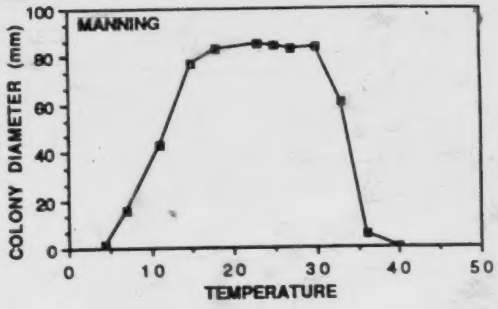
Temperature Gradient Studies

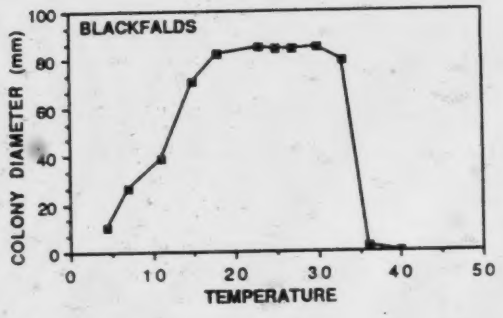
Although all isolates survived at 4.5 C, only the Manning and Olds isolates were able to grow at that temperature. In the upper range, all isolates grew at 33 C and all were able to survive at 36 C. Forty degrees was lethal to the Brooks, Olds and Manning isolates, while some regrowth occurred among the Blackfalds and Sexsmith isolates following the 6 day exposure to that temperature (Fig. 13).

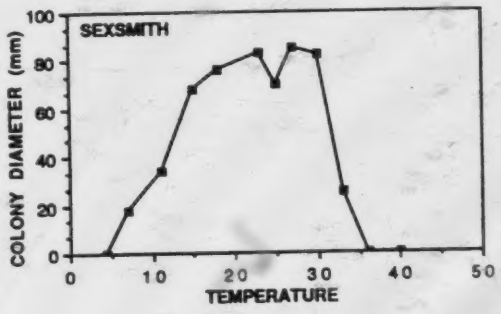
The effect of temperature on growth rate was determined by daily measurements of radial growth over a 6 day period at temperatures ranging from 20 to 40 degrees. The growth rate of each isolate was temperature dependent (Fig. 14). For example, Blackfalds displayed the most rapid growth at 23 C and reached the limits of the plate in only 5 days (Fig. 14a). Furthermore, this isolate was sensitive to cooler temperatures in its growth response, as evidenced from decreased growth at 20 C. In contrast, the Olds isolate displayed the fastest growth at 27 C, although the difference between the growth rate at 20 C and 27 C was not substantial (Fig. 14b).

Fig. 13. Temperature range for growth of 5 Cytospora leucostoma isolates on Malt Agar for 6 days. All isolates survived at temperatures of 4.5 C. Temperatures of 40 C were lethal to the Brooks, Olds and Manning isolates


BROOKS
COLONY DIAMETER (mm)
TEMPERATURE
0
10
20
30
40
50
0
20
40
60
80
100


MANNING
COLONY DIAMETER (mm)
TEMPERATURE
0
10
20
30
40
50
0
20
40
60
80
100


BLACKFALDS
COLONY DIAMETER (mm)
TEMPERATURE
0
10
20
30
40
50
0
20
40
60
80
100


SEXSMITH
COLONY DIAMETER (mm)
TEMPERATURE
0
10
20
30
40
50
0
20
40
60
80
100

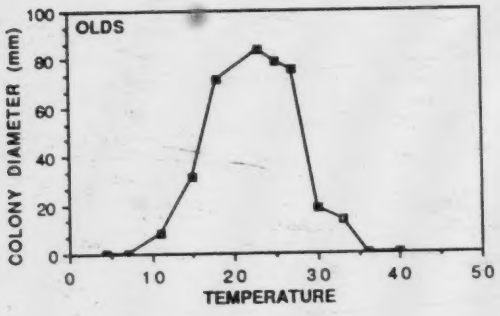
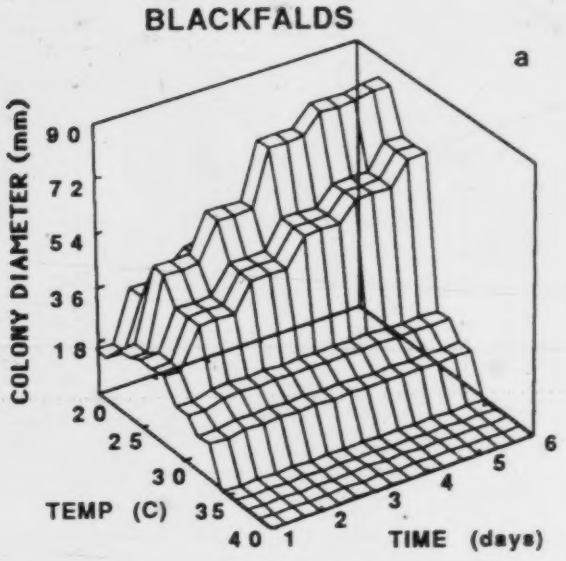
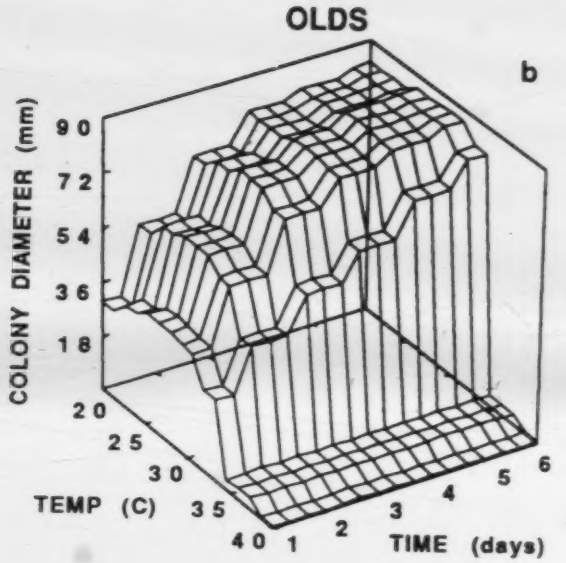
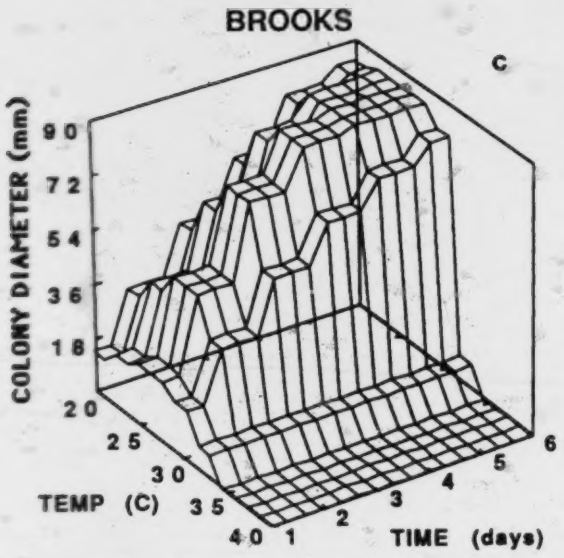
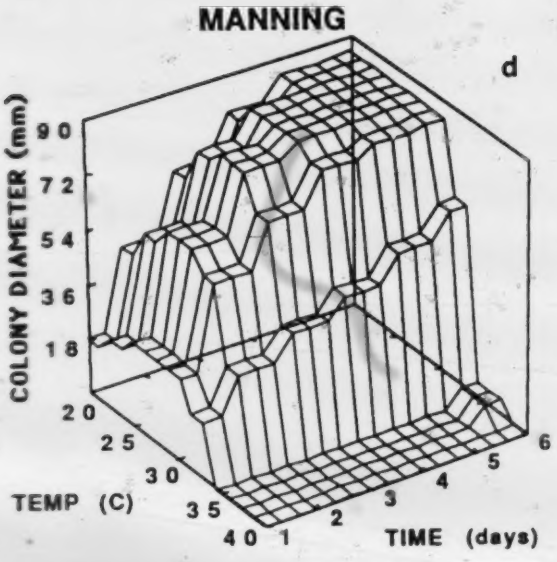

OLDS
COLONY DIAMETER (mm)
TEMPERATURE
0
10
20
30
40
50
0
20
40
60
80
100

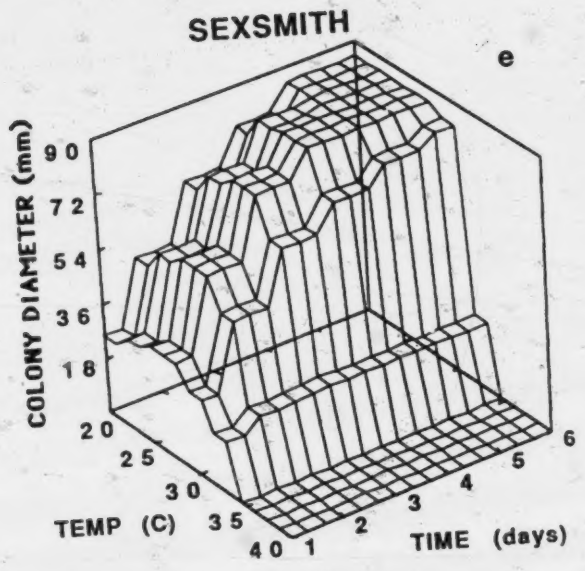
Fig. 14a-e. Growth response of 5 isolates of Cytospora leucostoma grown on Malt Agar as affected by temperature and time. Maximum growth area of a petri plate was 85 mm. F-value for the interaction of isolate x temperature x time was significant at the 0.01 level. (a) Blackfalds (b) Olds (c) Brooks (d) Sexsmith (e) Manning.


BLACKFALDS
a
COLONY DIAMETER (mm)
90
72
54
36
18
20
25
30
35
40
TEMP (C)
1
2
3
4
5
6
TIME (days)


OLDS
b
COLONY DIAMETER (mm)
90
72
54
36
18
20
25
30
35
40
TEMP (C)
1
2
3
4
5
6
TIME (days)


BROOKS
c
COLONY DIAMETER (mm)
90
72
54
36
18
20
25
30
35
40
TEMP (C)
1
2
3
4
5
6
TIME (days)


MANNING
d
COLONY DIAMETER (mm)
90
72
54
36
18
20
25
30
35
40
TEMP (C)
1
2
3
4
5
6
TIME (days)


SEXSMITH
e
COLONY DIAMETER (mm)
90
72
54
36
18
20
25
30
35
40
TEMP (C)
1
2
3
4
5
6
TIME (days)

DISCUSSION

In disease surveys, Cytospora spp. are frequently found in the absence of the teleomorphic stage, making it difficult to identify conclusively the species involved. The genus Cytospora is very uniform, characterized by hyaline, allantoid conidia and slender, branched phialidic conidiophores. Differences in conidial dimensions are negligible between species, rendering this characteristic unreliable in species diagnosis (Spielman, 1985).

The sexual stage was obtained only once in culture (Leonian, 1921) and attempts to repeat this have not been successful. Cultural characteristics have also proved unreliable as distinguishing features. Many discrepencies exist in the literature with respect to cultural descriptions, depending on the type of media on which the fungus was grown. In this study, very pronounced differences were observed with different media.

Cytospora colonies derived from single spores are often so variable that this cannot be used to identify the fungus. Lukezic et al. (1965) found significant differences in colonies from ascospores of L. persoonii originating from the same ascus. The appearance of some colonies corresponded to prior descriptions of L. cincta while others corresponded to L. persoonii. Helton and Konicek (1961) found that fungal reisolations from inoculated trees tended to develop triangular sectors, a trait not present in the original cultures. Similar results were obtained by Helton and Moisey (1955) using isolates from prune trees. In addition to sectoring, the reisolates also developed more erratic and rapid growth habits. The most reliable method of identification thus far has been an

association between the anamorphic and teleomorphic stages on host tissue (Speilman, 1985).

In this study, L. persoonii was found in association with the asexual form of the fungus at two locations. It was distinguished from the genus Valsa by the presence of a black delimiting zone line or conceptacle surrounding a well developed entostroma characteristic of Leucostoma species (Barr, 1978). Leucostoma persoonii was separated from L. cincta based on the absence of a single central pycnidium in the perithecial stroma, a key taxonomic feature of L. cincta (Proffer and Jones, 1989). Among the isolates observed in this study, the pycnidia always occurred within a stroma similar to, but separate from, the perithecial stroma. Those isolates which were not found in association with the sexual stage could only be presumptively identified as C. leucostoma. Spore measurements were very uniform, but as stated previously, fell within the range of both C. leucostoma and C. cincta. In culture, all isolates formed a thin hyphal mat with no aerial mycelium which initially varied in color, gradually becoming black. Pycnidia, formed on the surface, exuded a reddish-brown cirrhus characteristic of C. leucostoma (Willison, 1936).

The optimum growth temperature was 23 C to 27 C which is in accordance with the 28 C to 30 C optimum generally accepted for L. persoonii (Dhanvantari, 1969).

Evidence for strain differences was demonstrated by pairings of the five isolates and by the differential growth response on various types of media. A distinctive black zone line formed along the interface of advancing colony margins indicating the presence of genetically distinct mycelia (Coates et al., 1985). In compatible reactions, a continuous hyphal

mat is formed with no distinctive line of demarcation (Goldstein and Gilbertson,1981; Puhalla and Hummel, 1983). The Sexsmith, Brooks, Blackfalds and Olds isolates appeared to be self compatible. In self-pairings with the Manning isolate each colony maintained its own individual integrity. It could be that these self-pairings were actually compatible and the colonies did not merge due to self-inhibition of growth. The self-inhibition of growth could be caused by factors, such as the formation of staling products or unfavorable changes in the pH. It may be that a typically compatible reaction will occur if the inoculations are made closeby. Further studies are needed to elucidate the reason for this unusual behavior. It was obviously incompatible with the other isolates.

It is interesting to note that the sexual stage was predominant at the Manning location. This is in contrast to the other locations where the anamorphic stage was found almost exclusively. This cannot be attributed to advanced stages of the disease, as this orchard was very well maintained and had a very low incidence of disease. Environmental conditions or differences in daylength existing at this extreme northern location could possibly be influential in triggering transcription of genes controlling the sexual stage.

Cytospora leucostoma causing dieback and canker disease was found throughout Alberta saskatoon orchards. The incompatibility reactions observed among the five isolates suggests that they are genetically diverse and different strains. The significance of the unusual characteristics of the Manning isolate is not clear. A more extensive investigation of

compatibility reactions between ascospores of the same ascus, as well as from different asci, would provide greater insight into the mating behavior of this fungus.

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CHAPTER IV

OXALIC ACID PRODUCTION BY STRAINS OF *CYTOSPORA LEUCOSTOMA* FROM *AMELANCHIER ALNIFOLIA*

INTRODUCTION

Dieback and canker disease has recently been reported in Alberta on *Amelanchier alnifolia* Nutt. (Pluim *et al.*, 1989). This hardy, native shrub, commonly known as the saskatoon or serviceberry, is a member of the Rosaceae family. Because of its extreme winter hardiness, interest in this crop has been incresasing. There are now an estimated 80 ha devoted to the production of saskatoons in Alberta (Hauser, L., 1990, Personal Communication, Alberta Special Crops & Horticultural Research Center, Brooks, Alberta).

The disease is caused by *Leucostoma persoonii* (Nitschke) Hohnel [anamorph = *Cytospora leucostoma* (Pers.: Fr.) Sacc.]. It is similar to Cytospora canker, a well-known disease on peach, plum, prune, sweet cherry and apple (Helton and Konicek, 1961; Helton and Moisey, 1955; James and Davidson, 1971; Kable *et al.*, 1967; Luepschen *et al.*, 1979; Proffer and Jones, 1989; Regner *et al.*, 1987).

In spite of numerous reports of the disease, relatively little is known regarding the mechanisms of pathogenesis. Tsakade (1959) suggested that a toxin released by *L. persoonii* was responsible for altering cell membrane permeability resulting in tissue damage. Gairola and Powell (1971) found that the extracellular enzymes polygalacturonase, xylanase,

cellulase and phosphatidase were secreted in greater quantities by virulent isolates of Cytospora as compared with hypovirulent forms. Hampson and Sinclair (1973) suggested that xylem dysfunction at canker sites due to fungal invasion was largely responsible for disease symptoms.

Traquair (1987) demonstrated the production of oxalic acid by peach isolates of L. persoonii. Bipyramidal and prismatic calcium oxalate crystals similar to those described for Sclerotium rolfsii Sacc. (Punja and Jenkins, 1984) were found in both culture filtrate and peach bark tissues inoculated with L. persoonii. Oxalic acid was detected using gas chromatography. Oxalic acid is believed to act synergistically with cell wall degrading enzymes by sequestering calcium from the middle lamella of cell walls and lowering the pH to levels optimal for enzymatic activity (Bateman and Beer, 1965; Rao and Tewari, 1987; Traquair, 1987).

The objectives of this study were to determine the production of oxalic acid by isolates of C. leucostoma from Amelanchier alnifolia and to discuss possible mechanisms of pathogenicity.

MATERIALS AND METHODS

Oxalic Acid Determinations

Isolates of C. leucostoma were collected from diseased saskatoon bushes at 5 locations throughout Alberta (Brooks, Olds, Blackfalds, Sexsmith and Manning). Inoculum was produced by inoculating sterile saskatoon twigs with each of the 5 Cytospora isolates. The twigs were maintained in sterile glass petri dishes on Whatman No. 3 filter paper. The paper was kept moist with sterile distilled water. Oozing cirrhi from

pycnidia were removed with sterilized wooden skewers and placed in 10 ml sterile distilled water in a test tube to make a concentration of 1×10^4 spores/ml. The tubes were agitated gently to disperse the spores. Erlenmeyer flasks (125 ml) containing 50 ml potato-dextrose broth were inoculated with 1.0 ml of the spore suspension and placed on a shaker (Lab-Line Orbit Shaker No. 3520) at 125 rpm under ambient light conditions (approximately 5.5 uEm^{-2} sec^{-1} and 22 C). Mycelium was harvested every two days for 24 days. Changes in pH were recorded at each harvest. Mycelial dry weight was determined by vacuum filtering each sample through a tared Whatman no. 3 filter paper moistened with distilled water. The paper and mycelium were frozen in liquid nitrogen and lyophilized for 24 hr in a Virtis Freezemoble 6 Freeze Dryer No. 6211-0330 and the weight of the mycelium was recorded. The total volume of filtrate remaining at each harvest was also recorded. Three replicates for each isolate were used.

Oxalic acid production was determined by adding 15 ml of filtrate to 10 ml 0.1M calcium chloride-acetate buffer (pH 4.5) and allowing the mixture to stand overnight. The following day the solution was centrifuged at 10,000 rpm for 20 min in a Sorval Centrifuge (RC-5B) at room temperature. The pellet was washed with 5% acetic acid and centrifuged as above. After centrifugation, the pellet was dissolved in 50 ml 4N sulfuric acid and titrated at 80 C with 0.02N potassium permanganate. After adjusting for total volume of filtrate remaining at each harvest, oxalic acid was expressed in mg/g mycelial dry wt. Data was subjected to ANOVA and Duncan's Multiple Range Test was used for mean comparison.

Crystal Analysis

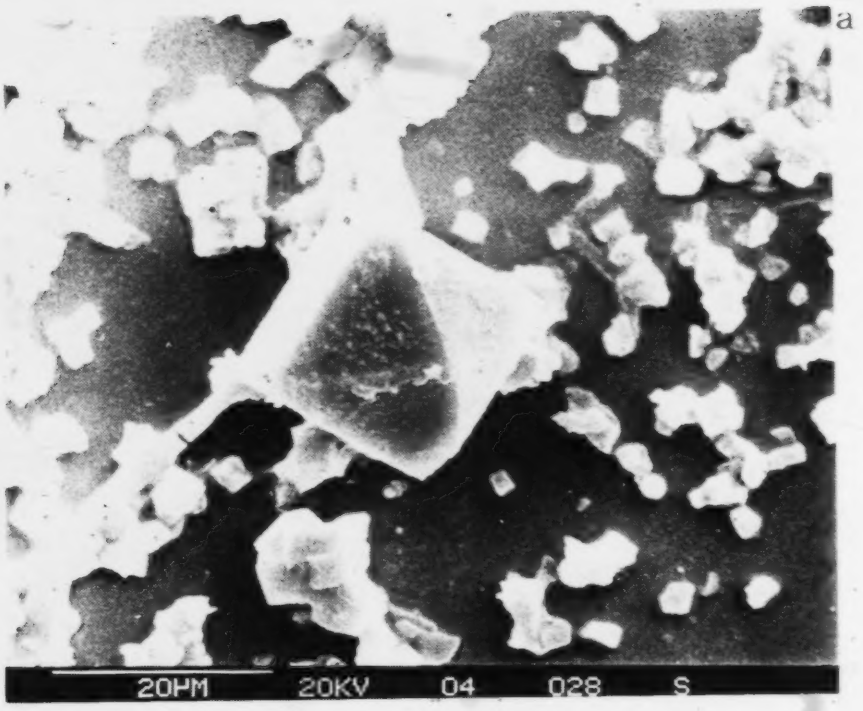
SEM and energy dispersive x-ray microanalysis were used to identify calcium present in crystals formed by C. leucostoma in liquid culture. Isolates were grown in potato-dextrose broth supplemented with 0.1% (w/v) calcium carbonate (PDBC). Following a two week incubation period, culture filtrate from the PDBC was centrifuged at 10,000 rpm for 20 min. The sedimented crystals were washed in 5% acetic acid and centrifuged twice following alternate rinses with sterile, deionized water. The crystals were then suspended in sterile water and a drop of this suspension was placed on aluminum stubs and air dryed. For studies on crystal morphology, specimens were gold coated following energy dispersive x-ray microanalysis. Micrographs were taken in a Cambridge Stereoscan 250 scanning electron microscope equipped with a Kevex Micro-X 7000 analyzer.

RESULTS

Crystal Analysis

Abundant bipyramidal crystal forms of the type described for calcium oxalate polyhydrate (Frey-Wyssling, 1981) were produced by all isolates during growth on PDA and PDBC. Numerous crystals of different sizes were observed in the culture media as well as inside the conidiomata (Fig. 1a-b). Pure crystals obtained from PDBC were not soluble in 5% acetic acid, indicating that they were not calcium carbonate or phosphate (Rao and Tewari, 1987). The x-ray emission spectra of the crystals confirmed the predominance of calcium (Fig. 2).

Fig. 1. (a). Scanning electron micrograph of calcium oxalate crystals produced by Cytospora leucostoma grown for 2 weeks in Potato Dextrose Broth supplemented with 0.1% calcium carbonate. Crystals were isolated directly from the liquid medium. (b). Scanning electron micrograph of calcium oxalate crystals observed within a pycnidium.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20HM 20KV 04 028 S

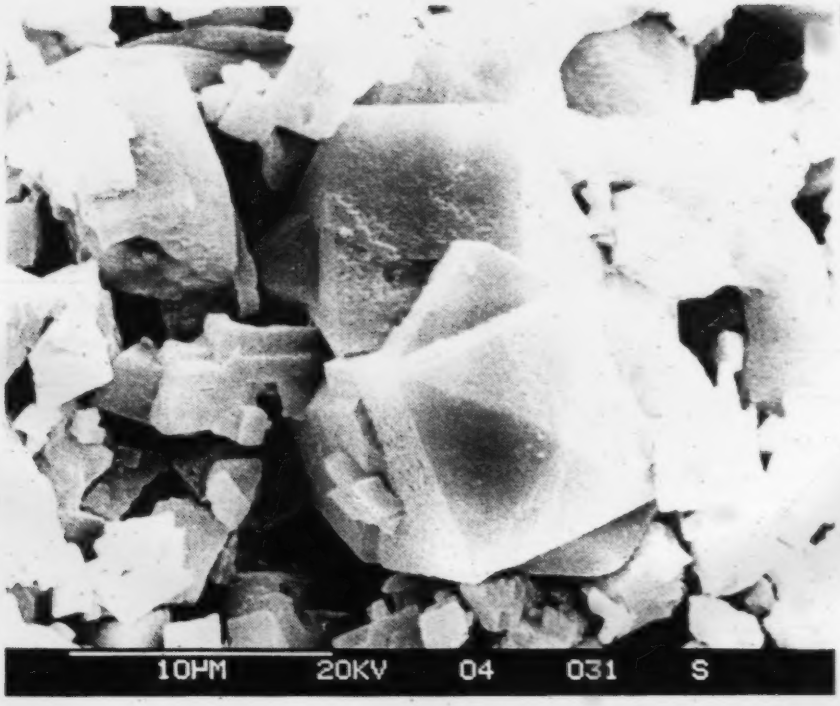
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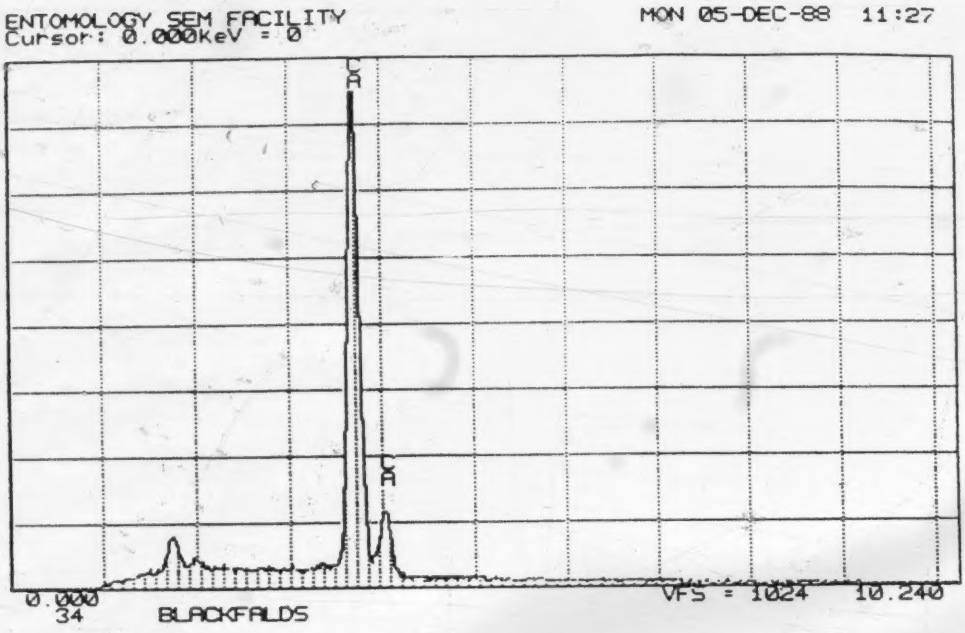
Fig. 2. Representative x-ray emission spectrum indicating the predominance of calcium in crystals formed in the presence of Cytospora leucostoma in Potato Dextrose Broth over a 24 day growth period.


ENTOMOLOGY SEM FACILITY
Cursor: 0.000keV = 0
MON 05-DEC-88 11:27
0.000
34
BLACKFALDS
VFS = 1024
10.240

Oxalic Acid Determinations

All isolates produced significant amounts of oxalic acid during the 24 day growth period. Oxalate was not detected in uninoculated PDB by $KMnO_4$ titration. The isolate x time interaction for oxalate production was significant at the 0.01 level, indicating that the isolates produced oxalic acid at different rates during growth. The average oxalate production by each of the 5 isolates over the 24 day growth period appears in Table 1.

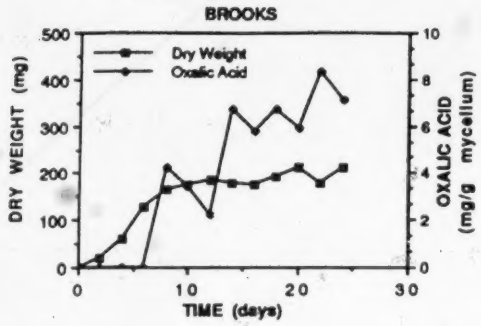
Generally, oxalic acid production increased with the age of the culture (Fig. 3). The onset of production varied with isolate. The first detectable levels of oxalic acid occurred on the sixth day of growth for the Olds and Blackfalds isolates, on the eighth day for the Brooks isolate, and on the twelfth day for the Manning and Sexsmith isolates. The pH of the media rapidly declined at the onset of growth and then gradually returned to the initial pH of 5.4. The first detectable levels of oxalic acid occurred at pH levels ranging from 2.6 to 4.0 and continued to increase even as the pH increased.

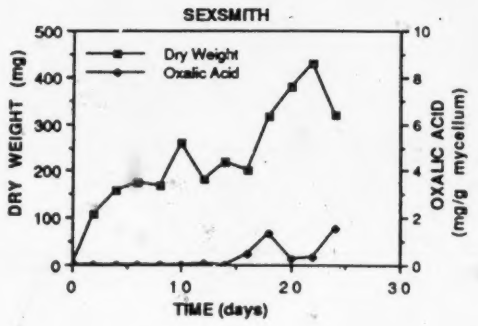
Table 1. Oxalic acid production from 5 isolates of Cytospora grown in Potato Dextrose Broth. The cultures were grown at 22 C for 24 days. Means represent average production over a 24 day period measured at 2 day intervals. F-value for the main effect of isolate was significant at the 0.01 level

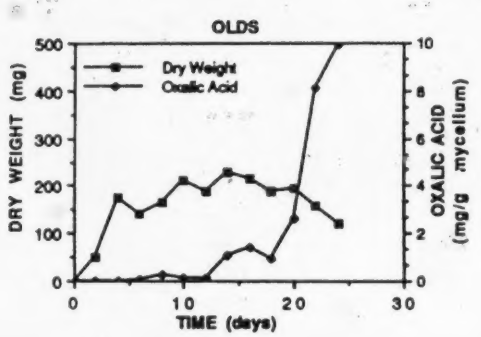
Isolate	Oxalic Acid mg/g mycelial dry wt
Brooks	4.21 a
Olds	2.10 b
Blackfalds	0.65 c
Sexsmith	0.43 c
Manning	0.33 c

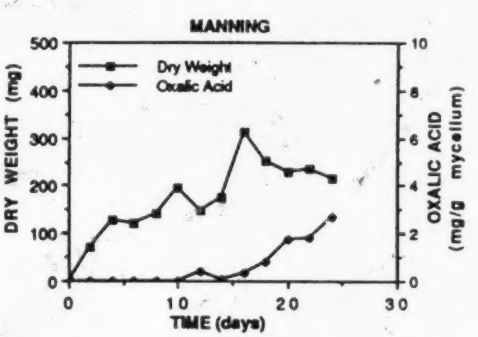
Mean separation by Duncan's Multiple Range test (P = 0.05)

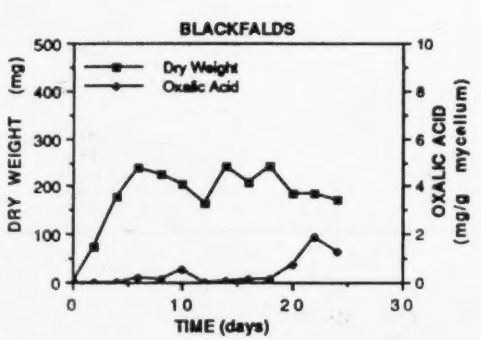
Fig. 3. Growth and oxalic acid production by 5 Cytospora leucostoma isolates grown in Potato Dextrose Broth at 22 C. Mycelial dry weight and oxalic acid levels were determined every 2 days. F-value for the interaction of isolate x time for dry weight and oxalic acid production were significant at the 0.01 level.


BROOKS
Dry Weight
Oxalic Acid
DRY WEIGHT (mg)
OXALIC ACID (mg/g mycellium)
TIME (days)


SEXSMITH
Dry Weight
Oxalic Acid
DRY WEIGHT (mg)
OXALIC ACID (mg/g mycellium)
TIME (days)


OLDS
Dry Weight
Oxalic Acid
DRY WEIGHT (mg)
OXALIC ACID (mg/g mycellium)
TIME (days)


MANNING
Dry Weight
Oxalic Acid
DRY WEIGHT (mg)
OXALIC ACID (mg/g mycellium)
TIME (days)


BLACKFALDS
Dry Weight
Oxalic Acid
DRY WEIGHT (mg)
OXALIC ACID (mg/g mycellium)
TIME (days)

DISCUSSION

Oxalic acid has been implicated as a factor in the pathogensis of Sclerotium rolfsii Sacc. (Punja and Jenkins, 1984; Bateman and Beer, 1965), Endothia parasitica (Murr.) And. (Havir and Anagnostakis, 1983) and Mycena citricolor (Berk. & Curt.) Sacc. (Rao and Tewari, 1987). It was recently found in the culture filtrate of L. persoonii and L. cincta, and calcium oxalate crystals were observed in association with hyphae colonizing inoculated wounds on peach twigs (Traquair, 1987).

In some fungi, oxalic acid may act synergistically with polygalacturonase by chelating calcium and lowering the pH to near optimum levels for tissue macerating enzyme activity (Bateman and Beer, 1965; Punja and Jenkins, 1984; Rao and Tewari, 1987, Traquair, 1987). The binding of calcium by oxalic acid permits macerating enzymes to hydrolyze the pectins in the middle lamella more readily. In S. rolfsii, oxalic acid production is known to occur early during the active growth phase of the fungus. This is accompanied by a drop in pH to 4.0, which is considered optimal for polygalacturonase activity (Bateman and Beer, 1965). In addition to polygalacturonase, Cytospora fungi are also known to produce cellulase, xylanase and phosphatidases in peach bark. Gairola and Powell (1971) found that virulent isolates produced significantly larger amounts of these enzymes than avirulent strains. Cellulase is believed to be important in the saprophytic phase of the fungus and in the later stages of pathogenesis. Xylanase is associated with the ability of the fungus to breakdown carbohydrate chains in xylan. The phosphatidases act on membrane

phospholipids causing an increase in membrane permeability (Gairola and Powell, 1971).

Results from the present study indicate that the onset of oxalic acid production was concomitant with an an initial decline in pH; however, pH then gradually increased over the 24 day growth period. Oxalic acid continued to be detected in increasing levels in spite of the increase in pH, suggesting that it was present as an oxalate salt in the filtrate. It is not clear from this study whether tissue macerating enzymes are an active part of pathogensis for Cytospora on saskatoons. Traquair (1987) found calcium oxalate crystals in the filtrate from 14 day old cultures of L. persoonii and in inoculated peach tissues after 10 days. However, the time of initial production and the accompanying changes in pH were not determined. Whether oxalic acid acts synergistically with cell wall degrading enzymes remains to be established for this fungus.

The symptoms observed on A. alnifolia do not suggest that enzymatic degradation is an active part of pathogenesis. Such symptoms include lesions with obvious tissue maceration that have a water-soaked appearance. On A. alnifolia, the diffuse cankers are characterized by cracked, exfoliated bark with dieback of tissues terminal to the canker location. Thus, the sequestration of calcium at the site of a canker could result in calcium deficiency to those portions of the branch distal to the canker. The deficiency symptoms would be most pronounced in the meristematic zones where cell divisions are occurring and calcium is required for the formation of a new middle lamella between daughter cells (Salisbury and Ross, 1978).

Hampton and Sinclair (1973) indicated that xylem dysfunction may be an important cause of symptoms in peach infections caused by C. leucostoma. They suggested that products of fungal metabolism which increase the viscosity of xylary fluid could impede cross transfer between vessels. Low levels of calcium were also found in leaves distal to cankers. Calcium is translocated in the xylem elements implying that it is being removed from xylem sap at canker sites. In saskatoons, branches with symptoms of dieback were always observed terminal to cankered areas. Thus, xylem dysfunction resulting in reduced calcium translocation or removal of calcium at canker sites may be a factor. Mohr and Watkins (1959) noted that tomatoes grown in soils with high calcium content were more resistant to infection by S. rolfsii than those grown in calcium deficient soils. Rao and Tewari (1988) demonstrated that external application of calcium to coffee leaves prior to inoculation by M. citricolor completely inhibited symptom development. Thus, removal of calcium at canker sites resulting in calcium deficiency to the terminal branches may also render the tissues more susceptible to infection by C. leucostoma.

The sequestration of calcium by oxalic acid may also affect the balance between cytosolic and extracellular calcium. Extracellular calcium, through calcium-pectates functions in cementing together adjacent cells (Jones and Lunt, 1967; Nooden and Leopold, 1988; Poovaiah, 1985). The divalent calcium cation is also important for stabilizing membrane structure through binding to negatively charged phospholipids on the exterior membrane surface. In addition to the positive effect of extracellular calcium on maintaining membrane integrity, cytosolic calcium

is known to stimulate plant senescence by binding to calmodulin which activates many lipolitic enzymes (Nooden and Leopold, 1988).

The lack of correlation between the oxalic acid produced by the isolates and pH does not necessarily rule out the possibility that oxalic acid production is involved in the pathogenicity of Cytospora fungi. An alternative hypothesis is that the oxalic acid produced sequesters significant amounts of extracellular calcium, destabilizing the membrane and increasing permeability. Extracellular calcium would thus permeate the destabilized membrane causing cytosolic levels to increase. The increase could activate calmodulin causing localized cell senescence through activation of lipolytic enzymes which catabolize membrane phospholipids. Notwithstanding the possibility that reduced pH from oxalic acid production enhances tissue macerating enzyme activity in some fungi, Cytospora spp. may cause disease symptoms by controlling cytosolic calcium levels of the host, thus using calcium in its role as a secondary messenger.

Cytospora spp. are known to be highly variable in pathogenicity ranging from virulent to saprophytic (Helton and Konicek, 1961). If oxalic acid is a factor in pathogenesis, then the differences in the amount produced, as well as the length of time required for the fungus to begin production, may influence the ability of the fungus to become established in tissues. Histological examination of tissues inoculated with C. leucostoma, elemental analysis of tissues distal to cankers and enzyme assays would be useful in determining the precise mechanisms of infection.

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CHAPTER V

SUMMARY AND CONCLUSIONS

Dieback and canker disease was present in all saskatoon orchards surveyed in Alberta in 1988 and 1989. The causal organism was identified as L. persoonii (Nitschke) Hohnel [anamorph = Cytospora leucostoma (Pers.: Fr.) Sacc.]. Characteristics of the organism were examined on host tissue as well as in culture. The anamorphic stage was found in association with the sexual stage in orchards at Manning and Olds. At both locations the teleomorphs were identified as L. persoonii. Those isolates that were found exclusively in an asexual stage, were identified as C. leucostoma based on appearance of sporocarps and spore dimensions in conjunction with temperature requirements which were close to the 28 C to 30 C optimum described for L. persoonii (Dhanvantari, 1969.).

Strain variation is apparent from growth media, oxalic acid and vegetative compatibility studies. The five isolates examined showed different growth rates in solid media as well as in broth culture. Significant isolate x time interactions were observed for oxalic acid production. However, the most conclusive evidence was incompatibility between isolates, suggesting that the strains belong to genetically distinct groups. Sources of variation in asexual reproduction include heterokaryosis, cytoplasmic inheritance and parasexualism (Agrios, 1979). The zone lines observed in the compatability studies imply that hyphal fusion, required for asexual exchange of genetic material, does

not occur. This may be significant in that dsRNA particles have recently been found in hypovirulent strains of L. persoonii and that transfer of these virus-like particles to aggressive strains may reduce virulence (Hammar et al., 1989) providing a means of biological control. However, if a large number of genetically incompatible groups exist and hyphal anastamosis cannot occur, then transfer of virus-like particles conferring hypovirulence may not be feasible. The results obtained with the Manning isolate, in which neither an incompatible or barrage reaction occurred, warrant further investigation. Additional studies involving pairings of cultures from single conidia and ascospores from the same sporocarp, from different sporocarps on the same canker and from different locations within an orchard are necessary to determine the extent of variation in C. leucostoma on A. alnifolia.

The aggressiveness of Cytospora fungi is known to vary from saprophytic to highly virulent (Helton and Konicek, 1961) indicating that physiologic strains, with varying degrees of pathogenicity and virulence, exist. Results from this study show that there were no differences between isolates in their effect on canker development following artificial inoculation. This conclusion must be regarded tentatively, as the cankers were evaluated after only 8 weeks. It is possible that significant differences would be seen after an extended period of time. Also, the inoculated bushes were maintained in an actively growing state. Luepschen et al. (1971) and Bertrand and English (1976) found that host response was an important limiting factor to canker development. Therefore, if the inoculated bushes were observed for an entire year, results may show significant differences. The production of oxalic acid was confirmed for

all isolates. The amount secreted by individual isolates, as well as the time of production onset, were significantly different. If oxalic acid is a factor in the pathogenicity of this fungus, then those isolates which produced oxalic acid earlier in the infection process and in greater quantities should be expected to be more virulent.

The exact role of oxalic acid in the pathogensis of L. persoonii has not been established. In Sclerotium rolfsii Sacc. it is known to act synergistically with polygalacturonase and some other enzymes by lowering the pH to optimum levels for enzyme activities (Bateman and Beer, 1965; Punja and Jenkins, 1984). In the present study, the lack of correlation between oxalic acid production and pH suggests that tissue macerating enzymes may not be directly involved. Hampson and Sinclair (1973) have suggested that calcium ions are removed from xylary fluid at cankered sites resulting in calcium deficiency to distal portions of the branches. Thus, calcium deficiency may be partially responsible for symptom development as well as rendering the host tissues more susceptible to fungal invasion. Calcium which binds to pectates in the middle lamella functions in cementing adjacent cells together. It is also responsible for normal membrane function in plant cells as a stabilizer of the phospholipid bilayer (Jones and Lunt, 1967; Salisbury and Ross, 1985). Mohr and Watkins (1959) and Rao and Tewari (1988), have shown that calcium application increases host resistance in tomato and coffee to infection by S. rolfsii and M. citricolor (Berk. and Curt.) Sacc., respectively. Further studies, including histological examination of cankered tissues, emzyme assays and

elemental analysis of diseased branches, are required to identify factors important in the pathogenesis of C. leucostoma.

The general impression from field surveys indicated that C. leucostoma is present in virtually all saskatoon growing areas of Alberta. Observations indicated that the severity of the disease may be related to conditions that weaken the plant resulting in a predisposition to disease. Studies designed to examine the influence of these factors on Cytospora infection would be useful in determining preventative measures.

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APPPENDIX A

REPORT OF SPECIES IDENTIFICATION FROM
C.A.B. MYCOLOGICAL INSTITUTE

ISOLATE	HERB. IMI NUMBER	IDENTIFICATION
Brooks	329183	Cytospora leucostoma (Pers.:Fr.) Sacc.
Olds	329184	Cytospora leucostoma (Pers.:Fr.) Sacc.
Blackfalds	329185	Cytospora leucostoma (Pers.:Fr.) Sacc.
Sexsmith	329186	Cytospora leucostoma (Pers.:Fr.) Sacc.
Manning	329187	Cytospora leucostoma (Pers.:Fr.) Sacc.